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Calmodulin-dependent Protein Kinase IV Regulates Hematopoietic Stem Cell Maintenance*[S]◆

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The hematopoietic stem cell (HSC) gives rise to all mature, terminally differentiated cells of the blood. Here we show that calmodulin-dependent protein kinase IV (CaMKIV) is present in c-Kit$^+$ Scal$^+$ Lin$^{-/low}$ hematopoietic progenitor cells (KLS cells) and that its absence results in hematopoietic failure, characterized by a diminished KLS cell population and by an inability of these cells to reconstitute blood cells upon serial transplantation. KLS cell failure in the absence of CaMKIV is correlated with increased apoptosis and proliferation of these cells *in vivo* and *in vitro*. In turn, these cell biological defects are correlated with decreases in CREB-serine 133 phosphorylation as well as in CREB-binding protein (CBP) and Bcl-2 levels. Re-expression of CaMKIV in *Camk4*$^{-/-}$ KLS cells results in the rescue of the proliferation defects *in vitro* as well as in the restoration of CBP and Bcl-2 to wild type levels. These studies show that CaMKIV is a regulator of HSC homeostasis and suggest that its effects may be in part mediated via regulation of CBP and Bcl-2.

The intracellular Ca^{2+} receptor calmodulin (CaM)[3] and its downstream CaM-dependent protein kinases (CaMK) I, II, and IV connect transient increases in intracellular Ca^{2+} with physiological processes such as proliferation, development, and differentiation. CaMKIV is a multifunctional serine/threonine (Ser/Thr) protein kinase found predominantly in cells of the brain, testis, thymus, and ovary as well as in mature T cells and neutrophils (1–7). This predominantly nuclear protein kinase regulates transcription mediated by several transcriptional activators including CREB (8), CBP (9, 10), MEF2 (11), RORα (12), and COUP-TF (12), in response to transient increases in intracellular Ca^{2+}.

To evaluate the physiological roles of CaMKIV, two independent C57BL/6J × 129Sv lines of *Camk4*$^{-/-}$ mice were generated using different targeting strategies (7, 13). Both lines of *Camk4*$^{-/-}$ mice revealed deficits in brain (4, 13, 14) and T cell function (1). Furthermore, targeted expression of a kinase-inactive CaMKIV in mice results in defective thymocyte survival and activation (2). Although the precise cascade of events in which CaMKIV participates remains enigmatic, neurons (13, 14) and memory T cells (1) null for *Camk4* show a marked decrease in CREB Ser^{133} phosphorylation (phospho-CREB), indicating that CREB-mediated transcription may contribute to the observed phenotypes. In addition, CaMKIV has been shown to phosphorylate CBP at Ser^{301}, thereby enhancing CREB-CBP-mediated transcription (10). Such findings have led to the hypothesis that a CaMK cascade, of which CaMKIV is a component, is a part of the pathway by which Ca^{2+} regulates transcription mediated by CREB and CBP (15).

In this report, we investigated whether CaMKIV is involved in early hematopoietic development and found that the absence of CaMKIV results in a reduction in the number of c-Kit$^+$ Scal$^+$ Lin$^{-/low}$ cells (KLS cells), a cell population that includes long-term and short term hematopoietic stem cells as well as other multipotent progenitor cells (16). Specifically, we found that the *Camk4* gene is expressed in KLS cells and that CaMKIV is required for KLS cells to repopulate the bone marrow in transplantation assays. Furthermore, *Camk4*$^{-/-}$ KLS cells display enhanced proliferation as well as increased apoptosis, *in vivo* and *in vitro*, compared with wild type (WT) cells and have decreased levels of phospho-CREB (pCREB), CBP, Bcl-2 mRNA and Bcl-2 protein. Re-expression of CaMKIV in *Camk4*$^{-/-}$ KLS cells restores Bcl-2 and CBP levels and rescues the proliferation defects. Thus, our data reveal a novel role for CaMKIV in the maintenance of hematopoietic homeostasis and suggest that this role involves suppression of inappropriate KLS cell proliferation.

EXPERIMENTAL PROCEDURES

Mouse Strains—The *Camk4*$^{-/-}$ mouse was generated using a CaMKIV target vector construct that deletes the first two exons of CaMKIV and the two known transcription initiation sites (7). Genotyping was performed as described previously (7). In the mixed genetic background of C57BL6/J × 129Sv, ~50% of the *Camk4*$^{-/-}$ pups showed growth retardation and died within the first 3 weeks of postnatal life. The remaining mice grew to adulthood but were infertile. Because these severe defects did not occur in the other line of *Camk4*$^{-/-}$ mice generated by Ho *et al.* (13), we initiated a breeding program for nine generations to stabilize the genetic background. This resulted in the loss of the fertility and premature death phenotypes but the brain and T cell phenotypes were maintained. All mice used in the present study were fertile, grossly asymptomatic and lived a normal life span. All animals were housed and maintained in the Levine Science Research Center Animal Facility located at Duke University under a 12-h light, 12-h dark cycle. Food and water were provided *ad libitum*, and all care was given in compliance within National Institutes of Health and institutional guidelines on the use of laboratory and experimental animals.

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[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Fig. 1.

◆ This article was selected as a Paper of the Week.

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[3] The abbreviations used are: CaM, calmodulin; CaMK, calmodulin-dependent protein kinase; CREB, cyclic AMP-responsive element-binding protein; CBP, CREB-binding protein; HSC, hematopoietic stem cells; KLS cells, cKit$^+$ Sca1$^+$ Lineage$^{-/low}$ cells; WT, wild type; FACS, fluorescence-activated cell sorting; MSCV, murine stem cell virus; GFP, green fluorescent protein; FITC, fluorescein isothiocyanate; PE, phycoerythrin; BrdUrd, bromodeoxyuridine; RT, reverse transcription; PI, propidium iodide; IRES, internal ribosomal entry site.

Bone Marrow Histology—Femurs isolated from 8-week-old mice were fixed in fresh 4% paraformaldehyde for 48 h, washed in 70% ethanol, and decalcified for 72 h. Glycol methacrylate infiltration and embedding were performed using JB-4 embedding kit (Polysciences, Warrington, PA). Two-μm sections were prepared and stained with hematoxylin and eosin.

White Blood Cell Differentials—Blood was extracted for analysis by cardiac puncture. Blood cell counts were performed using automated analysis on a System 9000 Automated Cell Counter (Serono-Baker Diagnostics, Allentown, PA).

Colony Forming Assays—Colony forming assays were performed by plating 1×10^4 unfractionated bone marrow cells in quadruplicate on Methocult methylocellulose medium (Stem Cell Technologies, Vancouver, British Columbia, Canada) and grown at 37 °C in 5% CO_2. The evaluation of colony forming units was performed after 2 weeks in culture as per manufacturer's protocol.

Isolation of Hematopoietic Stem Cells—Isolation of HSCs from bone marrow cells was performed using a FACSVantage (BD Biosciences) as described (16). In particular, HSCs were sorted based on positive expression of c-Kit and Sca-1 (c-Kit$^+$, Sca-1$^+$) and low/negative expression of the lineage markers (Lin$^{-/low}$).

Stem Cell Transplantation—Bone marrow transplants were performed using the congenic strain B6.SJL-Ptprc^a Pep3^b/BoyJ (CD45.1, Jackson ImmunoResearch Laboratories, West Grove, PA) as the recipient. Recipient mice, older than 10 weeks of age, were irradiated by exposing them to a single dose of 9.5 Gy ^{137}Cs source. The following day, c-Kit$^+$, Sca-1$^+$, Lin$^{-/low}$ hematopoietic stem cells (KLS cells) were isolated from 3-week-old wild type and *Camk4*$^{-/-}$ (both CD45.2) donor mice. About 1000 sorted KLS cells from one donor were injected into the retro-orbital sinus of five to six irradiated recipients, and the experiment was repeated with at least six WT and six *Camk4*$^{-/-}$ donors. The following day, bone marrow cells from three recipient mice per donor cell genotype were isolated and analyzed for the presence of CD45.2 marker to ensure "proper homing" of the donor KLS cells. To measure repopulation, peripheral blood was obtained from the retro-orbital vein every 3 weeks (17). The blood cells were labeled with CD45.1 FITC, CD45.2 PE, and either Mac-1 and Gr-1 for myeloid lineage or CD3 and B220 for lymphoid lineage analyses (16, 18).

Serial Bone Marrow Transplants—The primary recipient mice were sacrificed at 3.5 months. New CD45.1 recipient mice (n = 5/group) were irradiated (9.5 Gy in a single dose) and transplanted with 4×10^6 mononuclear bone marrow cells from sacrificed, individual primary recipient mice by injection via the retro orbital sinus. Bone marrow cells from each primary recipient were injected into five secondary recipients. Peripheral blood from secondary recipients was analyzed by flow cytometry every 3 weeks (17).

Bromodeoxyuridine (BrdUrd) Analysis—For *in vivo* BrdUrd labeling assays, WT and *Camk4*$^{-/-}$ mice were fed with 0.5 mg/ml of BrdUrd (Sigma) dissolved in drinking water for 4 days. KLS cells isolated from these mice were fixed in 70% ethanol at −20 °C, permeabilized, stained with BrdUrd-PE antibody according to manufacturer's protocol (Pharmingen), and analyzed by fluorescence-activated cell sorting (FACS) for the presence of BrdUrd-PE-positive cells. For Ki-67 labeling, approximately 10,000 freshly sorted KLS cells were fixed in 80% ethanol for 12 h at −20 °C, permeabilized with 0.1% Triton X-100 (Sigma), and stained with FITC-labeled Ki-67 antibody (Pharmingen) for 30 min. The cells were then washed and subjected to FACS analysis for the presence of Ki-67-FITC-positive cells.

AnnexinV Apoptosis Assay—Approximately 10,000 freshly isolated KLS cells were incubated with AnnexinV-FITC and propidium iodine according to manufacturer's instructions (Pharmingen). Stained cells were analyzed by flow cytometry within 30 min.

Immunocytochemistry—Freshly isolated KLS cells were collected onto slides by cytospin, either immediately after isolation or after stimulation with 2 μM ionomycin or 3 μM forskolin (both from EMD Biosciences, La Jolla, CA) for 10 min at 37 °C. The cells were fixed in 4% paraformaldehyde for 30 min and permeabilized using 0.5% Nonidet P-40 for 10 min. Following overnight incubation at 4 °C with either anti-CREB NT (rabbit polyclonal, Upstate, Charlottesville, VA), anti-phospho-CREB (against Ser^{133}, rabbit polyclonal, Upstate), anti-CBP (A-22, rabbit polyclonal, Santa Cruz Biotechnology, Santa Cruz, CA), anti-Bcl-2 (mouse monoclonal, Pharmingen), or anti-p21^{cip1} (C-19G, goat polyclonal, Santa Cruz Biotechnology) the slides were incubated with the appropriate fluorescent secondary antibody. Digital confocal images were taken of all samples with the same settings and analyzed using Metamorph® software to quantify the intensity of the fluorescence; $n > 50$ for each condition.

Real-time RT-PCR Analysis—Total RNA was prepared from ~10,000 freshly isolated HSCs using the RNAqueous-Micro kit (Ambion, Austin, TX), according to manufacturer's instructions. The first strand cDNA was prepared using SuperScript III reverse transcriptase (Invitrogen), according to manufacturer's directions. Quantitative real-time PCR-based gene expression analysis was performed using IQ SYBR Green Supermix with the respective primers, and the reactions were performed using a LightCycler (Roche Applied Science). The sequences of all the primers used in this study are available upon request.

Murine Stem Cell Virus (MSCV)-CaMKIV Add-back Experiments—CaMKIV-WT or CaMKIV-K71M cDNA was cloned into MSCV-IRES-GFP vectors, and high titer control and recombinant viruses were made by pseudotyping with vesicular stomatitis virus glycoprotein. Approximately 30,000 WT or *Camk4*$^{-/-}$ KLS cells were allowed to proliferate overnight at 37 °C in X-vivo-15 (Cambrex, Walkersville, MD) media supplemented with 2% fetal bovine serum, 30 ng/ml stem cell factor, 30 ng/ml Flt-3 ligand, and 50 μM 2-mercaptoethanol. The cells were infected with the appropriate MSCV virus at an MOI of 5:1 and were harvested 3 days after infection. Expression of CaMKIV-WT or CaMKIV-K71M was confirmed by RT-PCR using specific primers against CaMKIV. For *in vitro* cell proliferation assays, GFP$^+$-MSCV-infected KLS cells were FACS sorted at 15 cells per well into Terasaki plates. The cells were grown in X-vivo-15 (Cambrex, Walkersville, MD) media supplemented with 5% FBS, 30 ng/ml stem cell factor, 30 ng/ml Flt-3 ligand, and 50 μM 2-mercaptoethanol for 6 days. The proliferation rate of the KLS cells was estimated by counting the number of cells in each well at the indicated time points. For immunocytochemistry, GFP$^+$ virus-infected KLS cells were cytospun onto slides, fixed, and stained for respective antibodies as mentioned before. Protocols are available upon request.

RESULTS

Loss of CaMKIV Results in Diminished Bone Marrow Cellularity and Number of HSCs—Initial histological analysis of bone sections from adult *Camk4*$^{-/-}$ mice revealed a decrease in bone marrow cellularity (Fig. 1, *A* and *B*), raising the possibility that CaMKIV could play a role in hematopoiesis. To explore this idea, we analyzed peripheral blood samples drawn from Camk4$^{-/-}$ mice and found a 44% decrease in total white blood cells ($p < 0.005$), a 43% decrease in cells of the myeloid lineage (neutrophils, monocytes, and eosinophils; $p < 0.01$), and a 53% decrease in lymphoid cells (T and B cells; $p < 0.002$) compared with WT (Fig. 1*C*). To evaluate whether hematopoietic progenitor activity is compromised in the absence of CaMKIV, we performed colony forming

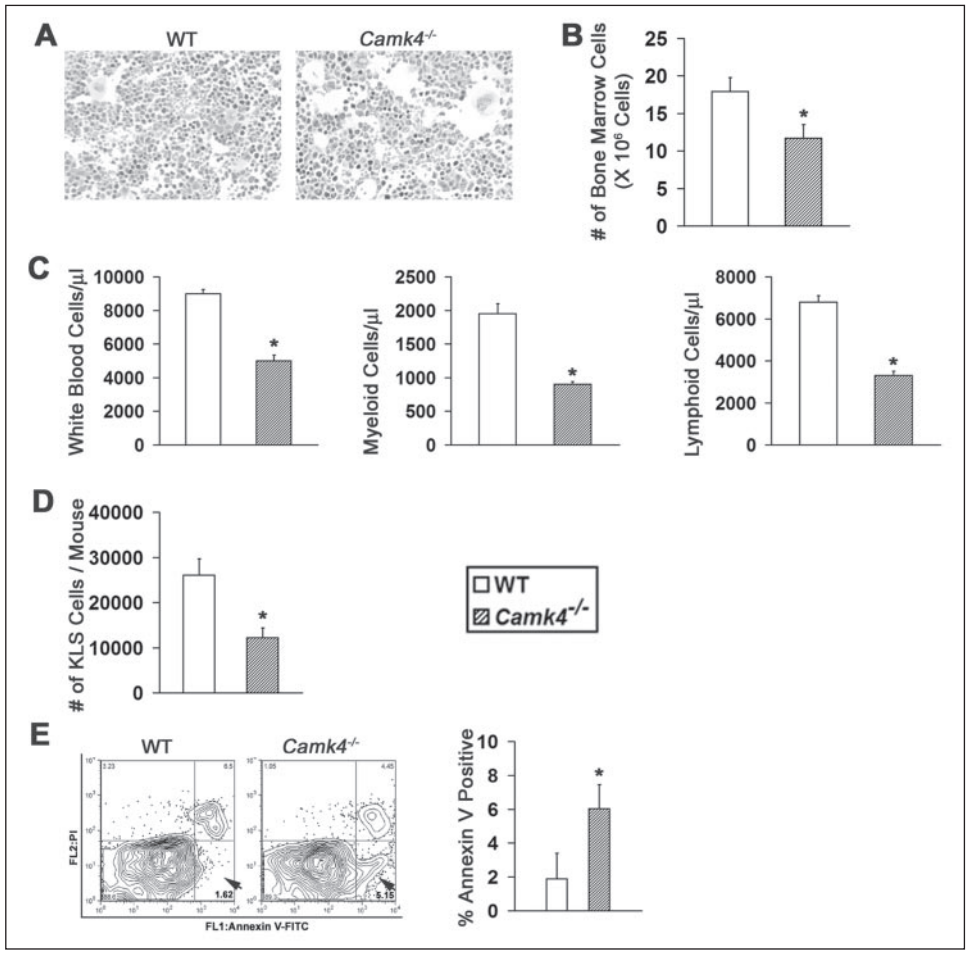

FIGURE 1. **Loss of CaMKIV leads to defective hematopoiesis.** *A*, sections of glycol methacrylate embedded femurs from WT or *Camk4*$^{-/-}$ mice were stained with hematoxylin & eosin and photographed (×100). *B*, *bar graph* depicting the average number of bone marrow cells per mouse in WT and *Camk4*$^{-/-}$ mice ($n = 18$ mice per genotype). * denotes statistical significance ($p < 0.05$). *C*, *bar graphs* showing hemocrit counts of white blood cells, myeloid cells (neutrophils, monocytes, and eosinophils), and lymphoid cells (B and T cells) in the peripheral blood of WT and *Camk4*$^{-/-}$ mice. Results represent the mean ± S.E., $n = 30$ mice. *D*, *bar graph* representing average number of KLS cells isolated per mouse in WT and *Camk4*$^{-/-}$ mice ($n > 30$). *E*, data representing results from AnnexinV:PI staining of freshly isolated KLS cells from WT and *Camk4*$^{-/-}$ mice. The *left panel* shows the FACS profile from a single representative experiment, and the *right panel* shows average values from $n = 3$. The *arrows* depict the AnnexinV^{+}PI^{-} population that represents the truly apoptotic cells. * denotes statistically significant differences; $p < 0.05$.

assays on bone marrow cells isolated from WT and *Camk4*$^{-/-}$ mice. Bone marrow cells from *Camk4*$^{-/-}$ mice formed fewer granulocyte-monocyte and pre-B cell colonies compared with the WT (data not shown) suggesting that CaMKIV might regulate progenitor cell development.

Long term and short term HSCs and the multipotent progenitors primarily reside in the bone marrow where they differentiate into committed progenitors in the myeloid and lymphoid lineages, which further mature before release into the peripheral blood. Thus, a reduction in the number of peripheral blood cells could result either from a primary defect in HSC self-renewal/maintenance or in differentiation of these cells. To distinguish between these two possibilities, we first examined the frequency of KLS cells in WT and *Camk4*$^{-/-}$ mice by FACS. Fig. 1*D* shows that there is a 2-fold reduction in the number of KLS cells (12,000 per mouse on average) in *Camk4*$^{-/-}$ mice compared with WT (26,000 per mouse on average). These results raised the possibility that CaMKIV could participate in the maintenance of the KLS cell population in the bone marrow. To determine whether the absence of CaMKIV resulted in a higher number of KLS cells dying by apoptosis (19), freshly isolated KLS cells from *Camk4*$^{-/-}$ and WT mice were stained for AnnexinV and propidium iodide (PI). While the AnnexinV-positive:PI-negative population only includes intact cells that are in the early stages of apoptosis, the AnnexinV-positive:PI-positive population includes cells that are necrotic or at advanced stages of apoptosis as well as cells killed or damaged during isolation. FACS analysis revealed three times more AnnexinV-positive:PI-negative (early apoptotic) cells in the freshly isolated *Camk4*$^{-/-}$ KLS cell population compared with WT (Fig. 1*E*). Thus, the lower number of KLS cells in *Camk4*$^{-/-}$ mice and the predisposition of these cells to apoptosis support the idea that CaMKIV has a role in maintaining KLS cell homeostasis by regulating their survival.

Camk4$^{-/-}$ *KLS Cells Are Compromised in Their Long Term Reconstitution Ability following Bone Marrow Transplantation*—To investigate whether the role for CaMKIV in KLS cells is cell autonomous and whether CaMKIV deficiency compromises long term HSC function, we performed *in vivo* bone marrow reconstitution assays by injecting irradiated CD 45.1 recipient mice with ~1000 KLS cells isolated from CD 45.2 WT or *Camk4*$^{-/-}$ donor mice (20–22). We chose to transplant KLS cells rather than whole bone marrow as the latter could skew the results due to the lower frequency of KLS cells present in *Camk4*$^{-/-}$ mice. We first analyzed bone marrow cells from recipient mice 19 h after transplantation and confirmed that donor-derived KLS cells from WT and *Camk4*$^{-/-}$ mice equivalently "home" to the bone marrow of the host mice (Fig. 2*Ai*). Next, recipient reconstitution was determined by FACS analysis of peripheral blood samples drawn every 3 weeks (Fig. 2, *Aii* and *B*). Mice transplanted with WT cells displayed normal reconstitution patterns at 3, 6, 9, and 12 weeks after transplant (16). In contrast, KLS cells from *Camk4*$^{-/-}$ mice led to significantly enhanced peripheral blood reconstitution between 3 and 6 weeks after transplant (Fig. 2, *Aii* and *B*). However, by 9 weeks the percentage of donor-derived CD 45.2 *Camk4*$^{-/-}$ cells was markedly reduced in the peripheral blood of recipient mice relative to WT cells (Fig. 2, *Aii* and *B*).

Next, to determine whether the *Camk4*$^{-/-}$ donor KLS cells that remained in the bone marrow at 12 weeks post-transplantation were still functional, we performed secondary bone marrow transplantation assays. Approximately, 4×10^6 total bone marrow cells (containing 6000 CD45.2 WT-derived KLS cells or 200 CD45.2 *Camk4*$^{-/-}$-derived

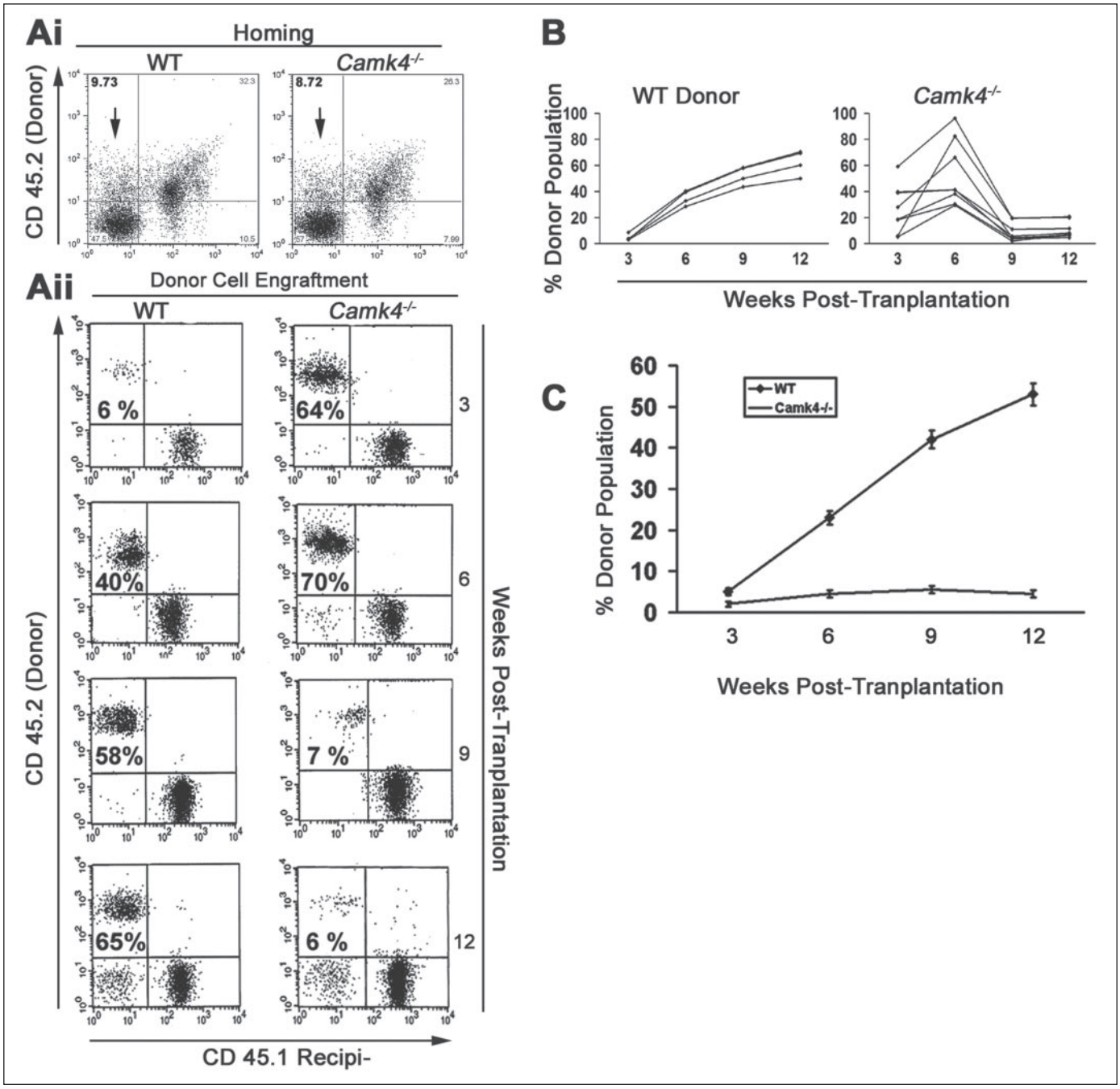

FIGURE 2. **Abnormal proliferation followed by premature exhaustion by Camk4$^{-/-}$ KLS cells during *in vivo* transplantation assays.** *Ai*, FACS analysis of homing to the bone marrow by donor CD45.2 WT and *Camk4*$^{-/-}$ KLS cells in irradiated CD 45.1 recipient mice, 19 h post-transplantation. Homing in a representative recipient for each donor genotype is depicted. The CD 45.1/45.2 double negative population (*lower right quadrant*) is the contaminating red blood cells in the bone marrow preparation. ***Aii*,** FACS analysis data from a representative experiment depicting reconstitution by WT or *Camk4*$^{-/-}$ donor KLS cells by a single representative recipient mouse at 3, 6, 9, and 12 weeks after bone marrow transplantation. *B*, *line graphs* representing reconstitution patterns of WT ($n = 4$) and *Camk4*$^{-/-}$ ($n = 8$) donor KLS cells at 3, 6, 9, and 12 weeks after transplantation by all the recipient mice in a representative reconstitution experiment ($n = 2$). Each line represents an individual irradiated recipient mouse. *C*, bone marrow cells from each primary recipient were transplanted into five irradiated secondary recipients and the percentage of cells from donor mice reconstituting at 3, 6, 9, and 12 weeks after transplantation were analyzed. Each line represents the mean ± S.E. for the indicated genotype ($n = 6$).

KLS cells) from recipient mice that had been transplanted 12 weeks previously with WT or *Camk4*$^{-/-}$ KLS cells were serially transplanted into new sub-lethally irradiated recipients. FACS analysis of blood samples drawn every 3 weeks showed no significant recipient reconstitution in mice transplanted with *Camk4*$^{-/-}$ cells, whereas reconstitution of WT cells occurred normally (Fig. 2*C*). Previous studies have shown that even as few as 1–10 viable long term HSCs are capable of reconstituting irradiated recipient bone marrow upon transplantation (18, 23). Cumulatively, these transplant data suggest that in contrast to the behavior of WT KLS cells, *Camk4*$^{-/-}$ KLS cells might inappropriately undergo a burst of engraftment followed by premature exhaustion, resulting in a loss of long term repopulating ability.

Absence of CaMKIV Results in Enhanced in Vivo and in Vitro Proliferation by KLS Cells and Re-expression of CaMKIV, but Not a Kinase-defective Mutant, Rescues This Defect in Vitro—The enhanced engraftment of *Camk4*$^{-/-}$ KLS cells early after the transplant could be attributed to an enhanced proliferation by the *Camk4*$^{-/-}$ KLS cells in the bone marrow of the irradiated recipient mice. To test this possibility

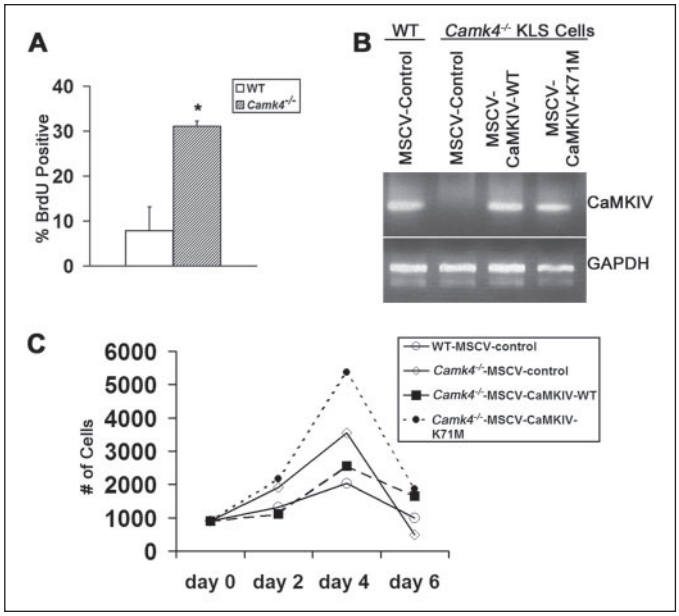

FIGURE 3. **CaMKIV inhibits inappropriate proliferation in KLS cells.** *A*, *bar graphs* depicting the percentage of BrdUrd-positive KLS cells in WT and *Camk4*$^{-/-}$ mice following *in vivo* labeling with BrdUrd for 4 days (*, $p < 0.05$). *B*, RT-PCR images showing the expression of CaMKIV in WT KLS cells and its introduction into *Camk4*$^{-/-}$ KLS cells through MSCV infection. Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was used as a loading control. *C*, MSCV-infected, GFP^{+} KLS cells were sorted into Terasaki plates at 15 cells/well and allowed to proliferate for 6 days. The numbers of cells at 0, 2, 4, and 6 days of *in vitro* growth from a representative experiment are depicted in the graph ($n = 3$).

we performed *in vivo* labeling of WT and *Camk4*$^{-/-}$ mice with BrdUrd for 4 days. The KLS cells were then isolated, fixed, stained with anti-BrdUrd antibody, and analyzed by FACS. While only 9% of the WT KLS cells were positive for BrdUrd incorporation, about 30% of *Camk4*$^{-/-}$ KLS cells stained positive for BrdUrd (Fig. 3*A*), indicating that a higher number of mutant cells are in proliferation. We also confirmed that *Camk4*$^{-/-}$ KLS cells have a greater proliferation index by Ki-67 labeling of freshly isolated KLS cells (data not shown). These results indicate that CaMKIV might act to suppress excessive proliferation by KLS cells in the bone marrow, thereby regulating HSC homeostasis.

If the role of CaMKIV in KLS cells is to suppress inappropriate cell proliferation, then re-expression of CaMKIV in *Camk4*$^{-/-}$ KLS cells should rescue their hyperproliferative phenotype. To test this idea we introduced either wild type CaMKIV (CaMKIV-WT) or a kinase-inactive CaMKIV (CaMKIV-K71M) into WT and *Camk4*$^{-/-}$ KLS cells using the MSCV-based vector that also encodes the GFP under the control of an IRES downstream of the cloned gene. KLS cells infected with the viruses were sorted based on GFP expression, and the expression of CaMKIV-WT and CaMKIV-K71M mRNAs was confirmed by RT-PCR (Fig. 3*B*). Equal numbers of GFP-positive WT and *Camk4*$^{-/-}$ KLS cells were then plated on Terasaki plates in media supplemented with 5% fetal bovine serum, 30 ng/ml stem cell factor and 30 ng/ml Flt-3 ligand. Proliferation was followed by counting the number of cells at 2-day intervals. On days 2 and 4 of *in vitro* growth, *Camk4*$^{-/-}$ KLS cells infected with MSCV-control virus proliferate at a higher rate (2-fold higher) than WT KLS cells (Fig. 3*C*). In addition, *Camk4*$^{-/-}$ cells are exhausted to a greater extent than the WT cells between days 4 and 6. Remarkably, re-expression of CaMKIV rescues both the hyperproliferation as well as the rapid exhaustion phenotypes characteristic of *Camk4*$^{-/-}$ KLS cells, and these cells behave in a similar fashion to WT cells infected with the control virus (Fig. 3*C*). CaMKIV activity is required for the rescue of the proliferation defects in the *Camk4*$^{-/-}$ KLS cells as the kinase-inactive mutant (K71M) was not able to alter proliferation of the mutant cells (Fig. 3*C*). In fact, not only did CaMKIV-K71M fail to reduce proliferation to WT levels but also hyperproliferation was exacerbated in the mutant cells, such that at 4 days of *in vitro* growth, there were more *Camk4*$^{-/-}$ cells expressing CaMKIV-K71M than *Camk4*$^{-/-}$ cells infected with the control virus. These data indicate that CaMKIV can regulate HSC proliferation *in vitro* and suggest that the enhanced engraftment observed *in vivo* is due to the overproliferation of *Camk4*$^{-/-}$ KLS cells. Cumulatively these findings indicate that CaMKIV activity is important for maintaining HSCs in a relatively quiescent state.

Lower Levels of Phospho-CREB, CBP, and Bcl-2 mRNA and Protein Levels in Camk4$^{-/-}$ *KLS Cells*—What is the signaling pathway by which CaMKIV functions to maintain hematopoietic homeostasis? CaMKIV can phosphorylate CREB on Ser^{133} (pCREB) in response to transient increases in intracellular calcium (24). Since decreased levels of pCREB have been found in neurons (13, 14) and memory T cells (1) of *Camk4*$^{-/-}$ mice, we examined whether decreased Ca^{2+}-induced pCREB was also observed in *Camk4*$^{-/-}$ KLS cells. As shown in Fig. 4, *Ai* and *Aii*, pCREB was reduced 2.5-fold in KLS cells deficient in CaMKIV as determined by immunofluorescence. In addition, whereas pCREB in WT cells was increased substantially following ionomycin treatment, very little increase was noted in similarly treated *Camk4*$^{-/-}$ cells (supplemental Fig. 1). As total CREB levels were similar in WT and *Camk4*$^{-/-}$ mice (Fig. 4, *Ai* and *Aii*) these results indicate that the Ca^{2+} signaling pathway leading to pCREB must be active in KLS cells *in vivo* and suggest that a defect in CaMKIV/pCREB-mediated transcription may compromise the functions of these cells.

Phosphorylation of CREB on Ser^{133} is required to recruit the CREB-binding proteins CBP or p300 to transcription complexes, which is in turn required for transcriptional activation of CRE-containing promoters (1, 9, 25, 26). In addition to phosphorylating CREB, CaMKIV has also been reported to phosphorylate CBP on Ser^{301} (10), which positively regulates its function as a transcriptional co-activator. Although antibodies specific to CBP-pSer^{301} that can be used in immunocytochemistry are unavailable, we did use a CBP polyclonal antibody to evaluate whether or not CBP levels might be altered in *Camk4*$^{-/-}$ KLS cells. Surprisingly, CBP is significantly reduced, by 2.4-fold, in the *Camk4*$^{-/-}$ KLS cells compared with WT cells (Fig. 4, *Ai* and *Aii*). These data raise the possibility that phosphorylation of CREB and/or CBP by CaMKIV might play a role in maintaining CBP levels in these cells and support the idea that a Ca^{2+}-dependent CaMKIV/CREB/CBP signaling cascade is active in HSCs.

If a CaMKIV signaling cascade functions through CREB and CBP to regulate transcription in HSCs, what target gene or genes might be activated to suppress proliferation as well as enhance survival of KLS stem cells? To explore possible mechanisms by which CaMKIV might regulate KLS proliferation and homeostasis, we compared the mRNA levels of several pro- and anti-apoptotic genes as well as the cyclin-dependent kinase inhibitor, p21^{cip1} in WT and *Camk4*$^{-/-}$ KLS cells (Fig. 4*C*). The absence of p21^{cip1} has previously been shown to result in hematopoietic stem cell exhaustion upon serial bone marrow transplantation (17). Our results reveal that, among the 14 mRNAs evaluated, only the Bcl-2 mRNA is differentially expressed between WT and *Camk4*$^{-/-}$ KLS cells, and as shown in Fig. 4, *B* and *C*, this 1.9-fold decrease in the *Camk4*$^{-/-}$ KLS cells is statistically significant. Several studies have shown that transcription of the pro-survival gene Bcl-2 gene requires pCREB and can be stimulated by Ca^{2+} (27–29). Moreover, in addition to its role in cell survival, Bcl-2 has been reported to play a role in maintaining cellular quiescence (30, 31). We also examined Bcl-2 protein levels in freshly isolated WT and *Camk4*$^{-/-}$ KLS cells by immunocytochemistry. Bcl-2 protein levels are 2.7-fold lower in

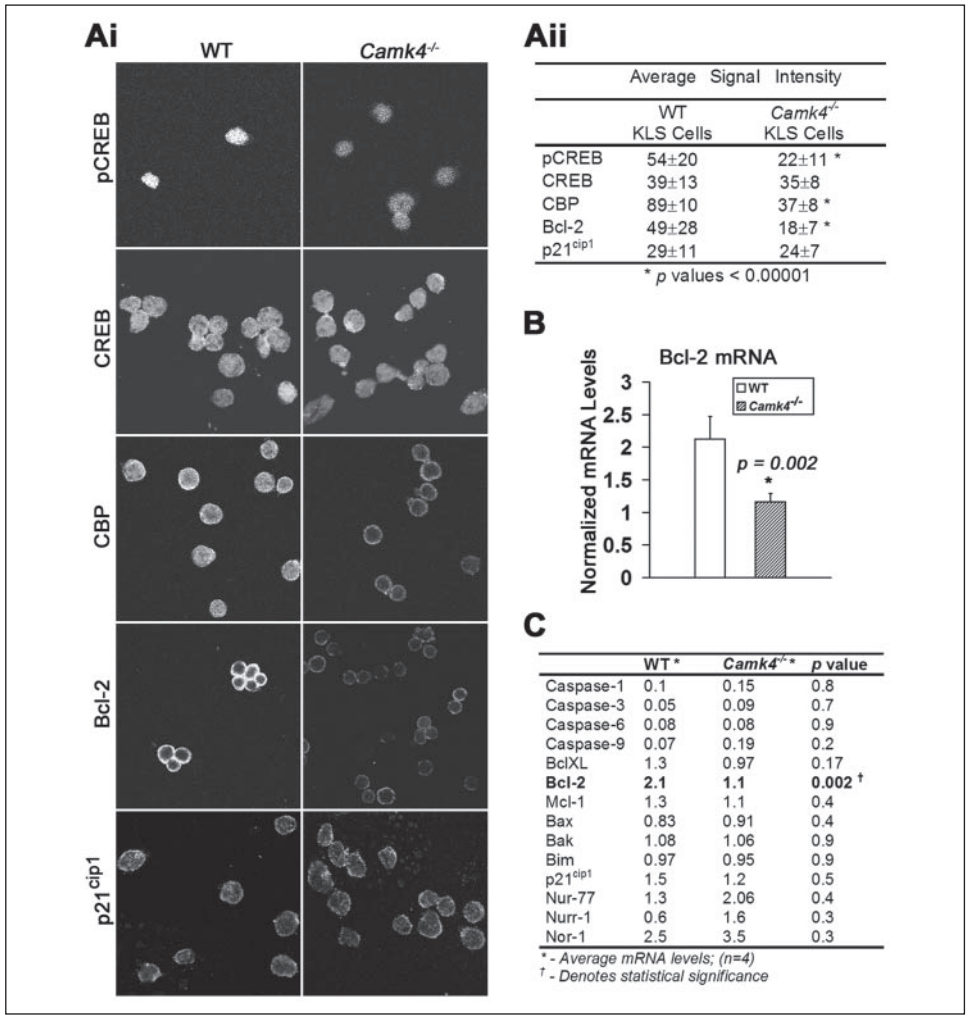

FIGURE 4. **Freshly isolated Camk4$^{-/-}$ KLS cells display decreased basal pCREB and CBP levels and decreased Bcl-2 mRNA and protein levels.** *Ai*, confocal fluorescent immunocytochemistry images depicting the levels of pCREB, total CREB, CBP, Bcl-2, and p21^{cip1} in freshly isolated KLS cells from either WT or *Camk4*$^{-/-}$ mice. Digital confocal microscopic images (630X) shown are representative of three experiments. *Aii*, table showing quantification of immunofluorescence signal intensity in cells stained for pCREB, total CREB, CBP, Bcl-2, and p21^{cip1} levels as depicted in *Ai*. Average ± S.E. of intensities measured from at least 50 cells from three experiments is shown. *B*, table depicting average mRNA expression levels of pro- and anti-survival genes as well as p21^{cip1} as analyzed by real-time PCR in freshly isolated WT and *Camk4*$^{-/-}$ KLS cells from four experiments, normalized to the respective glyceraldehyde-3-phosphate dehydrogenase levels and the respective *p* values. *C*, average Bcl-2 mRNA levels, based on real-time RT-PCR analysis, from freshly isolated WT and *Camk4*$^{-/-}$ KLS cells from four experiments, normalized to the respective glyceraldehyde-3-phosphate dehydrogenase levels, is shown. * denotes statistical significance ($p < 0.001$).

Camk4$^{-/-}$ KLS cells compared with the WT cells (Fig. 4, *Ai* and *Aii*), while protein levels of the cyclin-dependent kinase inhibitor p21^{cip1} are similar in WT and *Camk4*$^{-/-}$ KLS cells (Fig. 4, *Ai* and *Aii*). Collectively, our data indicate that in freshly isolated KLS cells, there is a positive correlation between the presence of CaMKIV, phosphorylation of CREB, and levels of CBP, Bcl-2 mRNA, and Bcl-2 protein.

Since freshly isolated *Camk4*$^{-/-}$ KLS cells show a 2.5-fold reduction in pCREB levels compared with WT cells, we wondered whether culturing these cells in the presence of stimuli that activate Ca^{2+}-independent CREB kinases would increase pCREB in *Camk4*$^{-/-}$ cells. Consistent with this idea, we could increase pCREB to the same level in freshly isolated *Camk4*$^{-/-}$ and WT KLS cells following incubation with forskolin (supplemental Fig. 1), demonstrating that the cAMP-dependent protein kinase pathway is intact and leads to CREB phosphorylation in both cell types.

Re-expression of CaMKIV Results in Restoration of WT Levels of CBP and Bcl-2 in Camk4$^{-/-}$ *KLS Cells*—We hypothesized that if the loss of CaMKIV in KLS cells is specifically responsible for decreased pCREB, CBP, and Bcl-2, then re-expression of CaMKIV in freshly isolated *Camk4*$^{-/-}$ KLS cells might reverse these defects by reconstituting the Ca^{2+}-dependent signaling pathway. As shown in Fig. 5, *A* and *B*, respectively, Bcl-2 and CBP levels were 3.5- and 2-fold lower in *Camk4*$^{-/-}$ KLS cells infected with MSCV-control virus, compared with control virus-infected WT cells. Re-expression of CaMKIV-WT, but not kinase inactive CaMKIV-K71M, quantitatively restores WT levels of CBP and Bcl-2 in the *Camk4*$^{-/-}$ KLS cells (Fig. 5, *A* and *B*). Interestingly, pCREB levels were only slightly reduced in *Camk4*$^{-/-}$ KLS cells infected with the control virus compared with WT cells and introduction of either CaMKIV-WT or CaMKIV-K71M resulted in only a slight but non-significant increase in pCREB levels in these cells (Fig. 5, *A* and *B*). We suspect that normalization of CREB phosphorylation in both cell types is due to serum-induced activation of CREB kinases other than CaMKIV as illustrated by the Forskolin experiments above (supplementary Fig. 1). These results also show that pCREB may be necessary but is not sufficient to restore Bcl-2 gene expression in the absence of CaMKIV and support the idea that an important component of the action of CaMKIV is an effect on CBP (9, 10). At any rate when taken together, our data support a role for a Ca^{2+}/CaMKIV/pCREB/CBP pathway in the regulation of Bcl-2 gene expression in KLS cells and strengthen our idea that this pathway may be important for promoting maintenance of HSC pool in mouse bone marrow by preventing inappropriate proliferation of KLS cells.

DISCUSSION

Self-renewing hematopoietic stem cells replenish billions of mature myeloid and lymphoid cells in the blood on a daily basis, a process that is vital for sustaining life. Understanding how the molecular regulation of HSC self-renewal is achieved is crucial for the improvement of HSC-based transplantation therapies. We have uncovered a novel role for CaMKIV in the maintenance of HSC homeostasis. The CaMKIV gene is expressed in KLS cells (Fig. 3*B*, *lanes 1* and *2*), and its absence results in lower numbers of these cells in the bone marrow of null animals. We

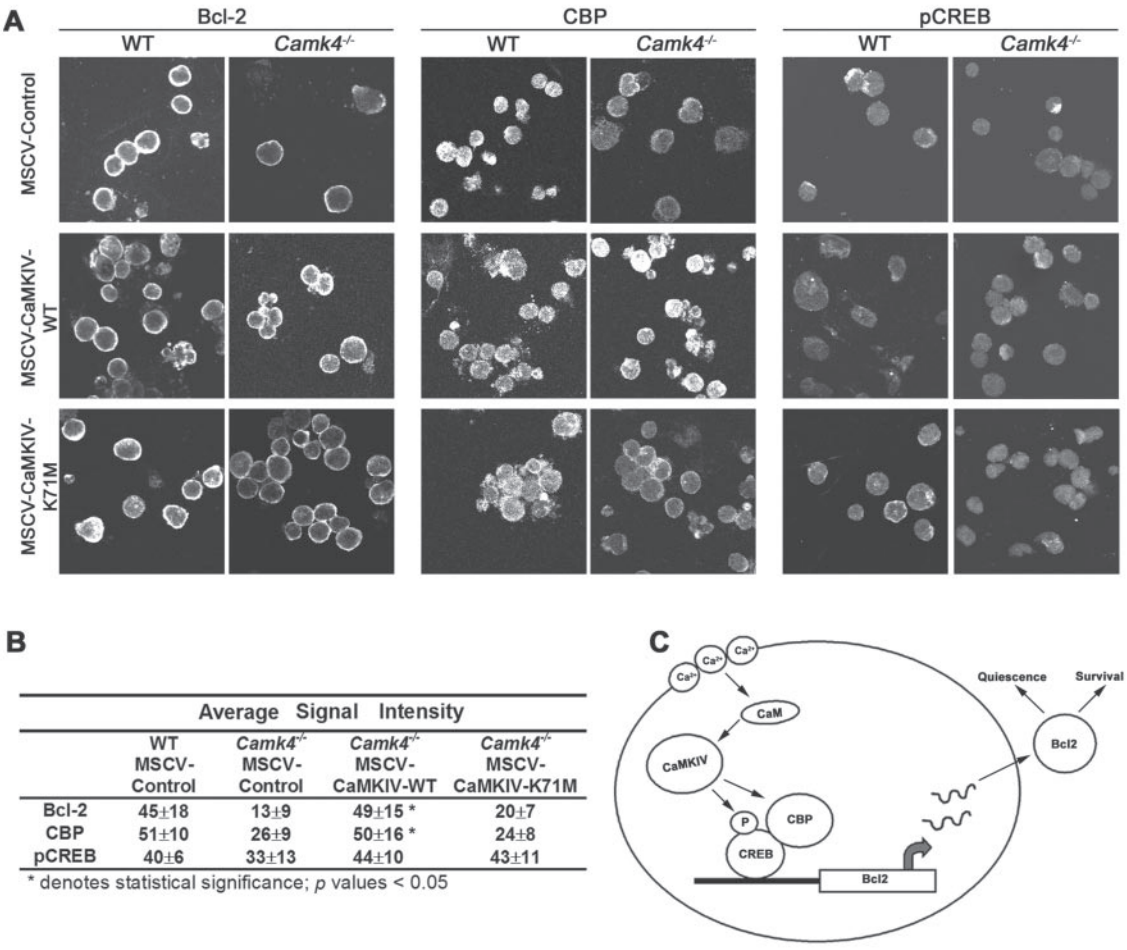

FIGURE 5. **Introduction of CaMKIV into Camk4$^{-/-}$ KLS cells results in elevation of Bcl-2, CBP, and pCREB protein levels.** *A*, confocal fluorescent immunocytochemistry images of WT and *Camk4*$^{-/-}$ mice KLS cells infected with MSCV-control, MSCV-CaMKIV-WT, or MSCV-CaMKIV-K71M depicting the levels of pCREB, CBP, or Bcl-2. Digital confocal images of ×630 magnification, representative of $n = 3$ experiments are shown. *B*, table showing average ± S.E. of fluorescence intensities from at least 500 MSCV infected cells from three experiments depicting the levels of Bcl-2, CBP, and pCREB. *C*, model depicting a possible mechanism for the regulation of HSC function by CaMKIV. In the HSC nucleus, Ca^{2+}/CaM-dependent CaMKIV causes elevation in pCREB and CBP, leading to the recruitment of CBP/pCREB-containing transcription complexes to the promoters of target genes such as Bcl-2, causing an elevation in Bcl-2 gene expression. The pro-survival protein Bcl-2, with an additional role in maintaining cellular quiescence, helps maintain HSC homeostasis in a CaMKIV-dependent manner.

also find that the absence of CaMKIV results in increased proliferation of KLS cells. When *Camk4*$^{-/-}$ KLS cells are challenged with expansion signals *in vivo* (bone marrow transplantation) or *in vitro* (growth factor-containing medium) they undergo premature proliferation followed by exhaustion. At least in culture, these altered proliferation defects could be rescued by re-expression of CaMKIV in the *Camk4*$^{-/-}$ KLS cells implying that, even acutely, this protein kinase serves as an inhibitor of inappropriate proliferation of the HSCs. The number of self-renewing HSCs in the bone marrow is regulated, at least in part, due to elimination of excess HSCs generated through inappropriate proliferation by apoptosis (32). Since the absence of CaMKIV results in increased proliferation of HSCs, perhaps due to their inability to maintain quiescence, as well as in predisposition of the cycling cells to exhaustion via apoptosis, we suggest that CaMKIV plays a role in HSC homeostasis.

The *Camk4*$^{-/-}$ mice utilized in this study are asymptomatic and live a normal life span when housed in a clean environment. The difference in the number of KLS cells between WT and *Camk4*$^{-/-}$ mice is 2-fold under homeostatic conditions, which may not be sufficient to cause overt immunological abnormalities. However, when challenged with stress or expansion signals such as those presented by bone marrow transplantation, these differences became much more important (Fig. 2, *A–C*). Similar results due to relatively small differences in KLS cell number under homeostasis that become amplified upon being challenged have been observed in Bcl-2 transgenic, p21$^{cip1-/-}$ and p18$^{ink4c-/-}$ mice (17, 32, 33). Additionally, the zinc finger transcriptional repressor *Gfi-1* was recently shown to be a regulator of HSC proliferation (34, 35). Although HSCs from both *Camk4*$^{-/-}$ and *Gfi-1*$^{-/-}$ mice show higher proliferation, unlike the *Camk4*$^{-/-}$ mice, *Gfi-1*$^{-/-}$ mice have a higher number of KLS cells in their bone marrow under homeostatic conditions (34, 35). However, similar to *Camk4*$^{-/-}$ KLS cells, *Gfi-1*$^{-/-}$ HSCs fail to reconstitute the bone marrow upon serial transplantation, due to their exhaustion in response to the expansion stimulus provided by transplantation (34).

Freshly isolated *Camk4*$^{-/-}$ KLS cells have significantly lower levels of pCREB, CBP, Bcl-2 mRNA, and Bcl-2 protein levels compared with WT cells. CBP and Bcl-2 levels could be restored by re-expression of CaMKIV, whereas culturing cells in the presence of serum and growth factors increased pCREB levels in KLS cells whether or not CaMKIV was present. These results suggest that pCREB may be necessary but is not sufficient for maintaining CBP and Bcl-2 in the absence of CaMKIV. pCREB is known to drive transcription from the CRE of the Bcl-2 gene promoter (28, 29), and CBP is the most important transcriptional co-activator for CREB. Thus, while it is likely that CaMKIV may also regulate other transcription factors present in KLS cells, our existing evi-

dence strongly suggests a role for a Ca^{2+}/CaMKIV/pCREB/CBP pathway in the regulation of Bcl-2 expression in KLS cells.

Consistent with the idea of a role for a CaMKIV, CBP, Bcl-2 pathway in the regulation of the HSC population, targeted overexpression of Bcl-2 in HSCs *in vivo* results in increased number, quiescence, and self-renewal of HSCs (32), precisely the opposite phenotypes that we report herein to arise due to the absence of CaMKIV. Thus, in the Bcl-2 transgenic mouse, although a higher percentage of HSCs is quiescent, the steady-state number of HSCs is actually increased as a result of a failure of these cells to be cleared by apoptosis (due to enhanced Bcl-2 expression) (32).

Mice haplo-insufficient for CBP also exhibit HSC exhaustion (36, 37). Although silencing of the CBP gene results in early embryonic lethality, mice heterozygous for CBP null mutation survive and display multiple severe phenotypes (37). Interestingly, CBP haplo-insufficient mice and CaMKIV null mice share phenotypic consequences in the brain and hematopoietic systems, although these two types of genetically altered mice do not phenocopy each other (36–38). One example of a difference between the two mouse lines is that the $CBP^{+/-}$ mice show age-dependent decrease in bone marrow cellularity and decrease in numbers of KLS hematopoietic stem cells as well as numbers of myeloid and B cell colony forming progenitors in the bone marrow (36). Second, HSCs from $CBP^{+/-}$ mice show no reconstitution only after tertiary bone marrow transplantation (37). Finally, unlike the case in *Camk4*$^{-/-}$ mice, the $CBP^{+/-}$ hematological defects only appear as the mice become older (36, 37). Regardless, both CaMKIV and a full complement of CBP seem to be required for maintaining HSC pools in the bone marrow.

Although our data indicate that CaMKIV may regulate the levels of CBP, it is not clear how this is achieved. Preliminary studies in cerebellar granule cells, which express both CBP and CaMKIV, show that CBP levels are also reduced in the absence of CaMKIV. However, expression of CaMKIV or incubation of these cells with proteosome inhibitors results in restoration of the CBP levels in *Camk4*$^{-/-}$ cells (data not shown). These results indicate that CaMKIV might regulate the stability of CBP in cells that express both proteins. Relevant to this idea CBP levels can be regulated by proteolysis as CBP polyubiquitination and degradation have been reported to occur in neurons undergoing degeneration in Huntington disease (39).

Based on this collective evidence, we suggest that a Ca^{2+}/CaMKIV/CREB/CBP signaling pathway is critical for the maintenance of HSC homeostasis and that one target for this pathway is likely to be the Bcl-2 gene (Fig. 5*C*). Unquestionably, additional genes regulated by this pathway collectively contribute to the regulation of HSC self-renewal by CaMKIV and we are actively pursuing their identity. Nevertheless, our data argue that decreased levels of Bcl-2, a protein with dual roles in the maintenance of cell survival and cell quiescence (31), may be an important contributing factor for the inability of the HSCs of the *Camk4*$^{-/-}$ mice to maintain quiescence and for the inappropriate proliferation of these cells when challenged with an expansion signal. In addition, the decrease in Bcl-2 in these proliferating HSCs might result in increased susceptibility of this cell population to apoptosis and together these events result in the eventual exhaustion of the hematopoietic stem cells.

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Calmodulin-dependent Protein Kinase IV Regulates Hematopoietic Stem Cell Maintenance

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