Regular Article

HEMATOPOIESIS AND STEM CELLS

CDK6 as a key regulator of hematopoietic and leukemic stem cell activation

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Key Points

- CDK6 acts as a transcriptional regulator to suppress *Egr1* in HSCs and LSCs, allowing their activation.
- *Cdk6*$^{-/-}$ HSCs fail to contribute to repopulation in competitive transplants, and BCR-ABL^{p210+} *Cdk6*$^{-/-}$ LSCs fail to inflict disease.

The cyclin-dependent kinase 6 (CDK6) and CDK4 have redundant functions in regulating cell-cycle progression. We describe a novel role for CDK6 in hematopoietic and leukemic stem cells (hematopoietic stem cells [HSCs] and leukemic stem cells [LSCs]) that exceeds its function as a cell-cycle regulator. Although hematopoiesis appears normal under steady-state conditions, *Cdk6*$^{-/-}$ HSCs do not efficiently repopulate upon competitive transplantation, and *Cdk6*-deficient mice are significantly more susceptible to 5-fluorouracil treatment. We find that activation of HSCs requires CDK6, which interferes with the transcription of key regulators, including *Egr1*. Transcriptional profiling of HSCs is consistent with the central role of *Egr1*. The impaired repopulation capacity extends to BCR-ABL^{p210+} LSCs. Transplantation with BCR-ABL^{p210+}–infected bone marrow from *Cdk6*$^{-/-}$ mice fails to induce disease, although recipient mice do harbor LSCs. *Egr1* knock-down in *Cdk6*$^{-/-}$ BCR-ABL^{p210+} LSKs significantly enhances the potential to form colonies, underlining the importance of the CDK6-*Egr1* axis. Our findings define CDK6 as an important regulator of stem cell activation and an essential component of a transcriptional complex that suppresses *Egr1* in HSCs and LSCs. (*Blood*. 2015;125(1):90-101)

Introduction

A cyclin-dependent kinase (CDK) is a critical regulator of cell-cycle progression, becoming activated upon binding to cyclins. Progression through the G_1 phase of the cell cycle is mediated by activation of the CDK4/6-cyclinD complex and subsequent phosphorylation of the retinoblastoma protein, which triggers E2F-dependent transcription.[1,2] CDK4 and CDK6 show 71% amino acid homology and have been considered to fulfill largely redundant functions because only the simultaneous deletion of both genes leads to embryonic lethality resulting from hematopoietic defects.[3,4] *Cdk6* deficiency is characterized by subtle defects in the hematopoietic system, such as defects in thymocyte development and a reduction in erythrocyte numbers.[4,5] CDK6 has been shown to have a kinase-independent function in myeloid cells, where it interacts with RUNX1 to block RUNX1-dependent transcription.[6] We recently discovered a key role for CDK6 in lymphoma formation: CDK6 transcriptionally regulates *Vegf-A* and *p16*^{INK4a} by interacting with signal transducer and activator of transcription (STAT) and AP-1 transcription factors.[7] A subsequent report described CDK6 as a transcriptional coregulator of nuclear factor κB p65.[8] CDK6 appears to have a key role in hematopoietic tumors, where it is frequently upregulated.[5,7] CDK6 has also been shown to be critical in acute myeloid leukemia (AML) and acute lymphoblastic leukemia driven by mixed lineage leukemia fusion proteins.[9,10] There is considerable interest in targeting CDK4/6 in cancer therapy, and the Food and Drug Administration nominated CDK4/6 inhibitors as the "breakthrough therapeutic advance" in 2013.

All hematopoietic cells arise from hematopoietic stem cells (HSCs), which possess the ability to self-renew and to differentiate into all blood cell lineages.[11] The existence of a deeply dormant HSC (*d*-HSC) population that only divides 5 times was recently postulated.[12] *d*-HSCs are activated in response to injury signals such as 5-fluorouracil (5-FU) treatment but are not thought to have any function in steady-state homeostasis.[12] Under stress conditions, *d*-HSCs enter the cell cycle, leading to a rapid increase in numbers of multipotent progenitors (MPP), which differentiate into distinct mature hematopoietic cells. The activation of quiescent *d*-HSCs is controlled by a network of transcriptional regulators such as EGR1, RUNX1, SCL, and PBX1.[13-16]

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The online version of this article contains a data supplement.

There is an Inside *Blood* Commentary on this article in this issue.

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Our understanding of the molecular networks that control HSCs is currently fragmentary. Research on leukemia provided the first striking observations for the existence of cancer stem cells. In AML and chronic myeloid leukemia (CML), only a limited population of cells is able to maintain and transduce disease. These so-called leukemic stem cells (LSCs) frequently express surface markers indicative of HSCs, and HSCs and LSCs clearly share functional characteristics and gene expression profiles.[17,18] LSCs and HSCs have unlimited capacity for self-renewal, and Bmi-1,[19] Wnt/β-Catenin,[20] and the Hedgehog pathway are activated.[21] There is evidence that LSCs may remain dormant for a long period (up to10 years),[22] explaining the high rate of relapse of leukemia patients after extended periods of remission. LSCs thus remain a major therapeutic challenge.

We describe a new facet of the regulation of HSCs and LSCs. *Cdk6*-deficient HSCs lack the ability to repopulate in competitive transplant experiments and fail to respond adequately to challenge with polyinosinic:polycytidylic acid (poly(I:C)) and 5-FU. Furthermore, $Cdk6^{-/-}$ BCR-ABL^{p210+} LSCs fail to repopulate upon transplantation. These results identify CDK6 as a crucial player in the activation of HSC and LSCs.

Methods

Mouse strains

All mice were maintained under pathogen-free conditions at the University of Veterinary Medicine, Vienna, Austria. $Ly5.1^+$($CD45.1^+$), wild-type, and $Cdk6^{-/-}$ (from M. Malumbres[4]) mice were kept on a C57Bl/6J background. *NOD/SCID/IL-2Rγ*$^{-/-}$ (*NSG*) mice were obtained from The Jackson Laboratory (USA). Six- to 8-week-old mice were used for experiments unless indicated elsewise. Animal experiments were performed in accordance with protocols approved by the Austrian law and the Animal Welfare Committee at the Veterinary University of Vienna.

Cell-cycle analysis

Cell-cycle analysis was performed in a 2-step protocol by staining with antibodies directed against HSC surface markers followed by intracellular staining with Ki-67 and 4,6 diamidino-2-phenylindole (DAPI). For intracellular staining, cells were fixed in Cytofix/Cytoperm (BD Biosciences), washed and stained with Ki-67 FITC (BD Bioscience), and then costained with 0.1 mg/100 mL DAPI in phosphate-buffered saline (PBS) with 0.1% Triton X-100 (Sigma-Aldrich).

Transplantation studies

For competitive transplantation assays, the bone marrow (BM) of the $Cdk6^{+/+}$($Ly5.1^+$) and $Cdk6^{-/-}$($Ly5.2^+$) mice were mixed at a ratio of 1:9, 1:1 or 9:1 and injected intravenously into lethally irradiated (9 Gy) C57Bl/6J mice (in total 5×10^6 cells/mouse). The irradiated control mice died after 9 days. For the assessment of long-term repopulation capacities of transplanted HSCs, mice were euthanized 16 weeks posttransplantation. BM, spleens, and peripheral blood were analyzed by fluorescence-activated cell sorter (FACS) for the contribution of $Ly5.1^+$ and $Ly5.2^+$ cells to individual LSK populations and mature lymphoid ($CD19^+$, $CD3^+$) and myeloid lineages ($Gr1^+$ $Mac1^+$).

Transcriptional profiling

Total RNA was extracted from the FACS fraction A cells ($Lin^-Sca1^+c\text{-}Kit^+$ $CD150^+CD48^-$) using the RNeasy Micro Kit (Qiagen). The RNA samples were quality controlled using the Laboratory-Chip technique (Agilent Bioanalyzer) and subsequently preamplified according to the TransPlex Whole Transcriptome Amplification WTA2 protocol (Sigma-Aldrich). Samples were then fluorescently labeled by in vitro transcription using the Two-Color Microarray-Based Gene Expression Analysis kit (Agilent) and hybridized onto Mouse Gene Expression G3 60K arrays (Agilent) containing ~56,000 60-mer probes. Images were acquired and quantified by confocal scanner and software (Agilent G2505C and Feature Extraction). Expression levels were processed using standard methods of normalization and significance analysis as described previously.[23] A multiple testing correction with false discovery rate adjustment by the Benjamini-Hochberg method was performed. Gene ontology and pathways were analyzed using Ontologizer,[24] JASPAR,[25] and GeneMANIA databases.[26] Heatmaps were generated using Caleydo software.[27]

Statistical analysis

Data are reported as mean values ± standard deviation and were analyzed by GraphPad. Differences were assessed for statistical significance by Student *t* test or 1-way analysis of variance. Kaplan-Meier plots were analyzed by the log-rank test. Statistical significance is as follows: $*P < .05$, $**P < .01$, $***P < .001$, $****P < .0001$.

Homing assay

Competitive setting. $Cdk6^{+/+}$ and $Cdk6^{-/-}$ BM cells were seeded on GP^+E86 retroviral producer cells (pMSCV-IRES-GFP or pMSCV-IRES-dsRed) in Dulbecco's modified Eagle medium containing 25 ng/mL IL-3, 50 ng/mL IL-6, 50 ng/ml stem cell factor (SCF), and 7 μg/mL polybrene. After 48 hours incubation, equal numbers (100 000 cells/mouse) of $Cdk6^{+/+}$ $dsRed^+$ LSKs and $Cdk6^{-/-}$ GFP^+ LSKs were injected intravenously into lethally irradiated (9 Gy) $Cdk6^{+/+}$ $Ly5.2^+$ animals together with 3×10^6 LSK–depleted BM carrier cells. After 18 hours, mice were euthanized and BMs were analyzed for the presence of $dsRed^+$ and GFP^+ LSKs.

Noncompetitive setting. $Cdk6^{+/+}$ $Ly5.2^+$ and $Cdk6^{-/-}$ $Ly5.2^+$ BM was sorted by FACS, and 1×10^6 cells (containing comparable numbers of LSKs) were injected into lethally irradiated $Cdk6^{+/+}$ $Ly5.1^+$ mice. After 18 hours, mice were euthanized and the BM was analyzed for the presence of $Ly5.2^+$ LSKs by FACS.

Results

Cdk6$^{-/-}$ HSCs fail to repopulate efficiently in a competitive transplant setting

CDK6 is expressed at high levels in hematopoietic cells, including HSCs (http://biogps.org/). To investigate the role of CDK6 in HSCs, we set up competitive transplant experiments using $Cdk6^{-/-}$ mice. Distinct ratios of $Cdk6^{+/+}Ly5.1^+$ and $Cdk6^{-/-}Ly5.2^+$ BM cells were transplanted into lethally irradiated $Ly5.1^+$ mice (Figure 1A). Sixteen weeks later, the contribution of $Cdk6^{+/+}Ly5.1^+$ and $Cdk6^{-/-}Ly5.2^+$ cells to the individual hematopoietic cell populations were analyzed (supplemental Figure 1A, available on the *Blood* Web site). Even when only a small proportion of donor cells were $Cdk6^{+/+}$, the majority of the leukocytes in the BM were derived from them (Figure 1B). Analysis of individual hematopoietic lineages revealed no $Cdk6^{-/-}/Ly5.2^+$ $Gr1^+Mac1^+$ or $CD19^+$ and only low numbers of $CD3^+$ cells (which most likely represent long-lived T cells) (supplemental Figure 1B). The spleen and blood contained very low numbers of $Cdk6^{-/-}/Ly5.2^+$ cells (data not shown). Remarkably, there were significant numbers of $Cdk6^{-/-}$ LSK ($Lin^-Sca1^+c\text{-}Kit^+$) cells in the BM, although they did not contribute to the pool of differentiated cells (Figure 1C). There were no detectable changes in the distribution of the LSK pool in recipient mice: the proportions of long-term HSCs, short-term HSCs, and MPP reflected the total LSK population (Figure 1D). Competitive (ratio 1:1) and noncompetitive homing experiments confirmed that the results could not be attributed to homing defects in $Cdk6^{-/-}$ LSKs (Figure 1E).

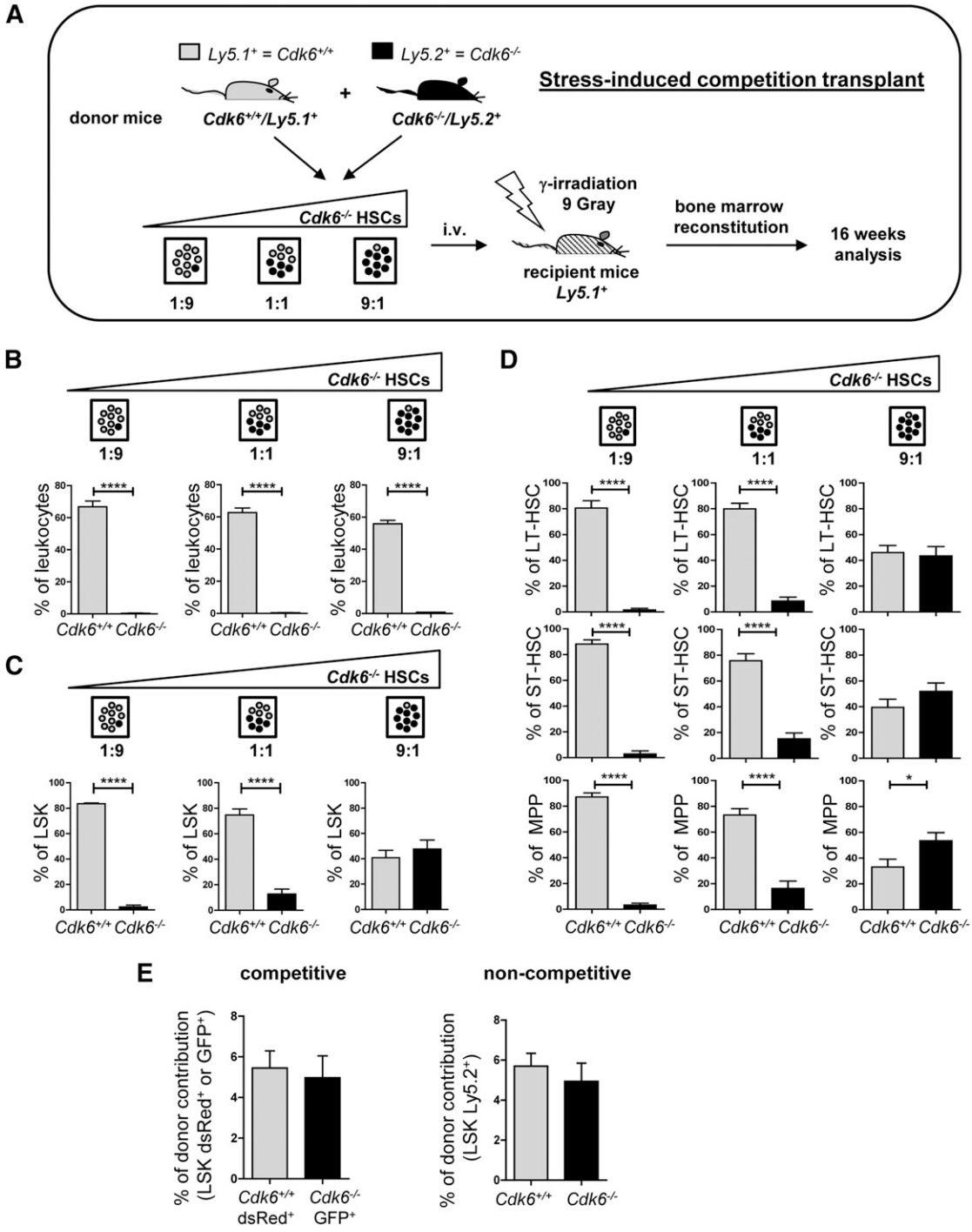


Figure 1. CDK6 is required for reconstitution of the hematopoietic system. (A) Schematic representation of experimental design. *Cdk6*$^{+/+}$*/Ly5.1*$^{+}$ and *Cdk6*$^{-/-}$*/Ly5.2*$^{+}$ BM cells were transplanted in ratios of 1:9, 1:1, and 9:1 into lethally irradiated (9 Gy) wild-type recipient mice. Long-term BM reconstitution was analyzed 16 weeks after transplantation (n = 5 per genotype; *Cdk6*$^{+/+}$ and *Cdk6*$^{-/-}$ groups were compared using Student *t* test). (B) Ly5.1^{+}/Ly5.2^{+} compositions were analyzed in total BM leukocytes (n = 5 per genotype; ****$P < .0001$). The leukocytic population included lymphocytes and myeloid cells, but excluded debris and erythrocytes, as determined by forward scatter/side scatter blots. (C) LSK cells were analyzed for Ly5.1^{+}/Ly5.2^{+} composition in each transplantation setting (n = 5 per genotype; ****$P < .0001$). (D) Contributions of Ly5.1^{+} and Ly5.2^{+} cells in long-term HSCs (LT-HSCs), short-term HSCs (ST-HSCs), and MPP (n = 5 per genotype; ****$P < .0001$, *$P < .05$). (E) *Cdk6*$^{+/+}$ and *Cdk6*$^{-/-}$ BM were infected with empty dsRed^{+} or GFP^{+} retrovirus. Equal numbers of *Cdk6*$^{+/+}$ dsRed^{+} LSKs and *Cdk6*$^{-/-}$ GFP^{+} LSKs (100 000/mouse) were injected in a 1:1 ratio into lethally irradiated recipient animals in a competitive setting (LSK-depleted carrier BM was added and a total of 3×10^6 cells/mouse were injected). After 18 hours, mice BM was analyzed for dsRed^{+} and GFP^{+} LSKs (left panel, n = 6). In a noncompetitive setting, equal numbers of either *Cdk6*$^{+/+}$*/Ly5.2*$^{+}$ or *Cdk6*$^{-/-}$*/Ly5.2*$^{+}$ BM cells were injected into lethally irradiated *Ly5.1*$^{+}$ mice (1×10^6 cells/mouse). After 18 hours, BM was analyzed for the presence of donor-derived *Ly5.2*$^{+}$ LSKs (right panel, n = 4 per genotype).

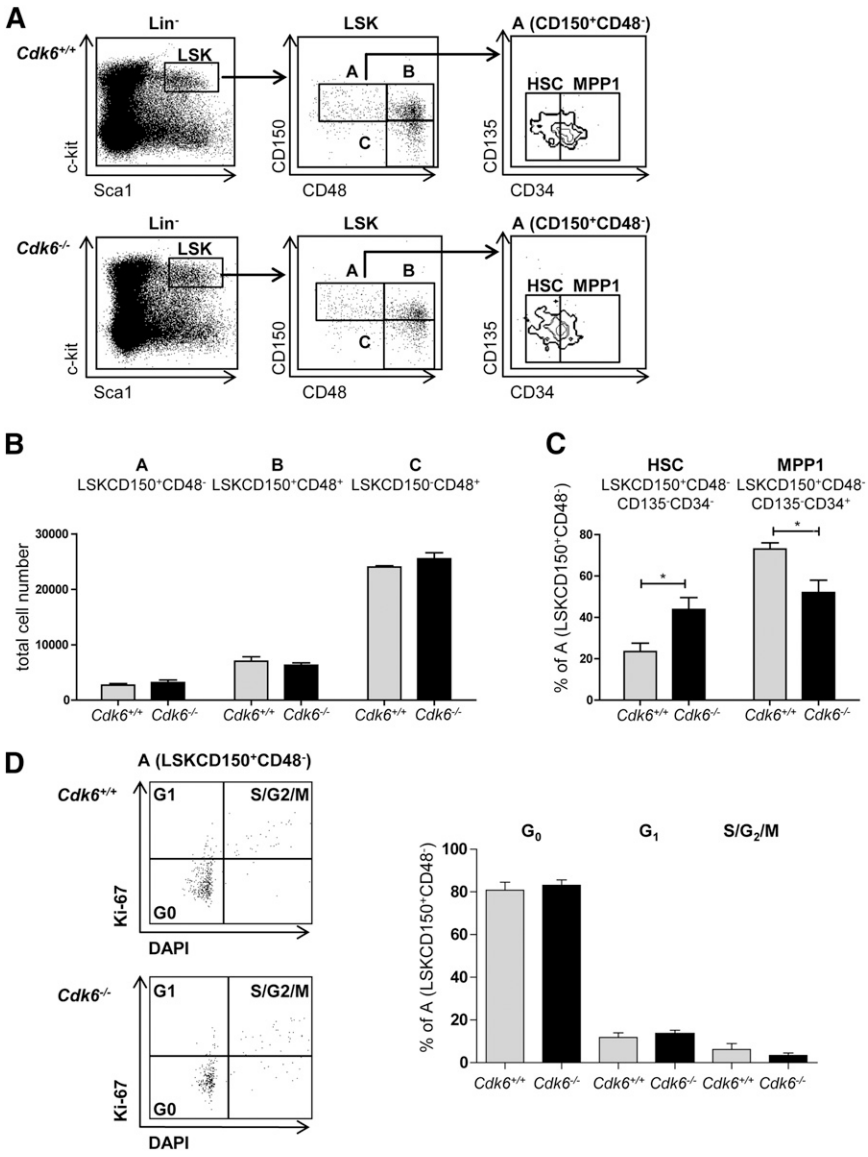


Figure 2. Increase in the most quiescent HSC population in *Cdk6*$^{-/-}$ mice. (A) LSK BM cells are subdivided into 3 populations based on CD150 and CD48 expression, LSKCD150^{+}CD48^{-} (A), LSKCD150^{+}CD48^{+} (B), and LSKCD150^{-}CD48^{+} (C). The LSKCD150^{+}CD48^{-} (A) population can be further subdivided into CD135^{-}CD34^{-} (HSC) and CD135^{-}CD34^{+} (MPP1) subsets. Sets of representative FACS blots of *Cdk6*$^{+/+}$ and *Cdk6*$^{-/-}$ BM cells are shown. (B) Total cell numbers of LSKCD150^{+}CD48^{-} (A), LSKCD150^{+}CD48^{+} (B), CD150^{-}CD48^{+} (C) in *Cdk6*$^{+/+}$ and *Cdk6*$^{-/-}$ animals are shown (n = 3 per genotype). (C) Analysis of individual subpopulations of LSKCD150^{+}CD48^{-} (A) cells is depicted. Population A is further subdivided into HSC and MPP1 (n = 3 per genotype; **P* < .05). (D) Cell-cycle distributions of fraction A cells were analyzed with DAPI and Ki-67 staining. One representative FACS blot per genotype is depicted (left). Summary of data (right; n = 6 per genotype).

Cdk6$^{-/-}$ mice harbor increased levels of quiescent HSCs

Under homeostatic conditions, the numbers of cells in the BM of *Cdk6*$^{-/-}$ animals was comparable to those of controls (supplemental Figure 2A)[4] and the total LSK numbers were unaltered (supplemental Figure 2B). Examination of individual LSK subpopulations using SLAM markers[12,28] (Figure 2A) failed to reveal significant alterations in the distribution of cells in fraction A (LSKCD150^{+}CD48^{-}), fraction B (LSKCD150^{+}CD48^{+}), and fraction C (LSKCD150^{-}CD48^{+}) (Figure 2B). However, the subdivision of fraction A cells into MPP1 (LSKCD150^{+}CD48^{-}CD34^{+}CD135^{-}) and HSCs (LSKCD150^{+}CD48^{-}CD34^{-}CD135^{-}) uncovered a significant increase of HSCs, the most quiescent fraction of LSKs, accompanied by a decrease in the percentage of MPP1 cells in *Cdk6*$^{-/-}$ mice (Figure 2C). The enhanced representation of the most quiescent stem cells in fraction A (LSKCD150^{+}CD48^{-}) was accompanied by a higher expression of Tie2 (supplemental Figure 2C). Under homeostatic conditions, the majority of cells in fraction A are in the G_0 phase,[12] and we failed to detect any significant differences in the cell-cycle profile of *Cdk6*$^{+/+}$ and *Cdk6*$^{-/-}$ fraction A (Figure 2D), fraction B, or fraction C cells (supplemental Figure 2D). Furthermore, we observed no significant differences in numbers of apoptotic cells in LSK and fraction A cells between genotypes (supplemental Figure 2E).

Enhanced lethality of *Cdk6*$^{-/-}$ mice upon repeated 5-FU treatment

The chemotherapeutic drug 5-FU kills dividing cells but spares dormant HSCs, which are subsequently forced to proliferate to repopulate the BM.[29] *Cdk6*$^{-/-}$ mice tolerated only 2 cycles of 5-FU application. In contrast, only 1 of 5 *Cdk6*$^{+/+}$ mice succumbed to repeated 5-FU treatment within 65 days, with the remainder surviving until the experiment was terminated after 150 days (Figure 3A-B). After a single dose of 5-FU, the percentage of cells in fraction A (which includes the most quiescent stem cells) was significantly enhanced in *Cdk6*$^{-/-}$ animals within 8 days (Figure 3C). More of the cells in fraction A from the *Cdk6*$^{-/-}$ mice remained in the G_0 phase (Figure 3D), indicative of their reduced ability to exit quiescence. No significant alterations in the proportion of cells in the G_0 phase were detectable in cells in fractions B and C (supplemental Figure 3A). Eight days after 5-FU injection, the numbers of Gr1^{+}Mac1^{+}, CD19^{+}, and Ter119^{+} cells were markedly lower in *Cdk6*$^{-/-}$ mice

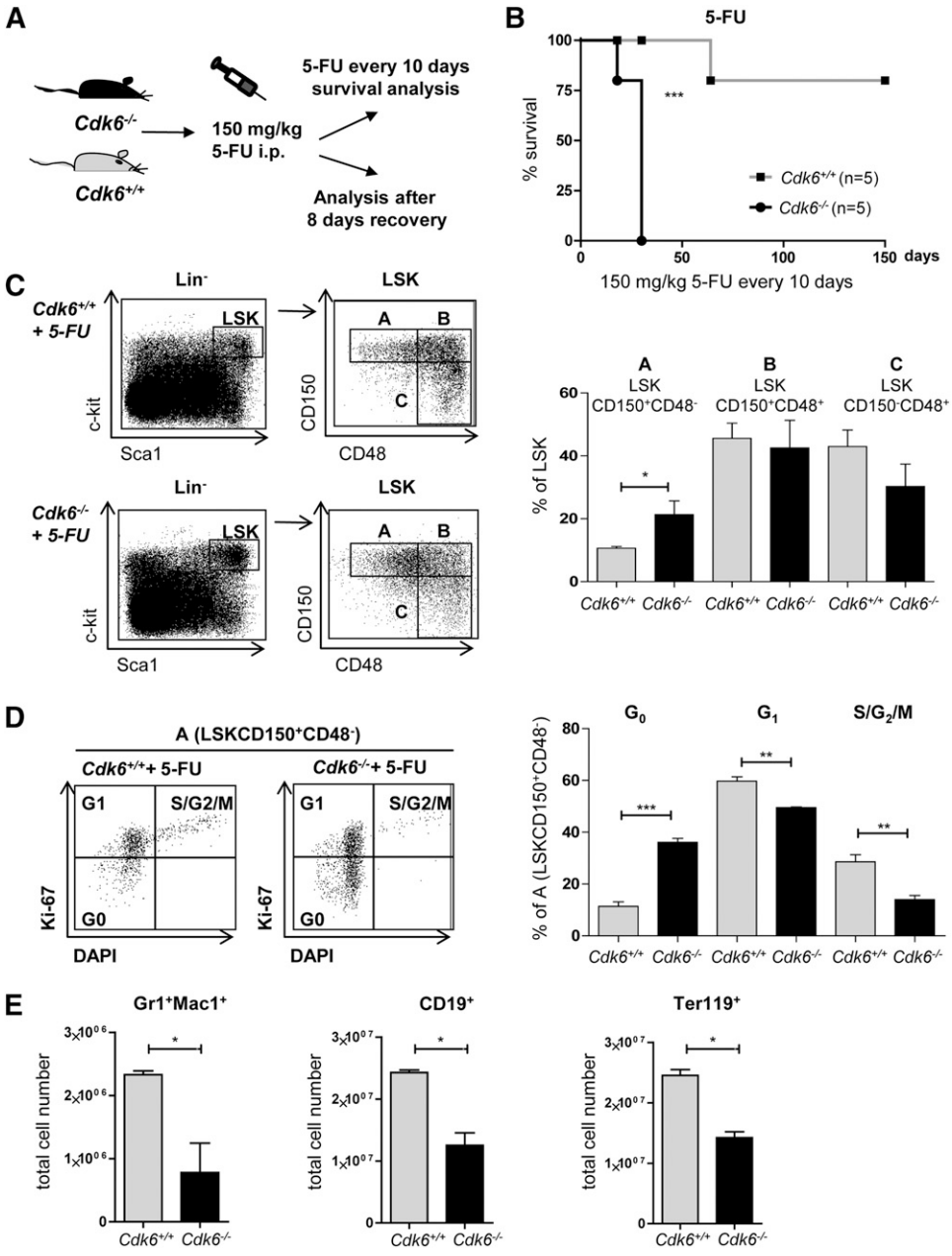


Figure 3. Impaired recovery of *Cdk6*$^{-/-}$ mice after 5-FU treatment. (A) *Cdk6*$^{+/+}$ and *Cdk6*$^{-/-}$ mice were repeatedly treated with 150 mg/kg 5-FU every 10 days (Figure 3B) or analyzed 8 days after a single dose of 5-FU (Figure 3C-E). (B) Four of 5 *Cdk6*$^{+/+}$ animals survived for 150 days, whereas all *Cdk6*$^{-/-}$ animals died within 25 days (n = 5 per genotype; ***$P < .001$). (C) Eight days after 5-FU treatment, BM of *Cdk6*$^{+/+}$ and *Cdk6*$^{-/-}$ mice was analyzed via FACS for the presence of LSKs and LSKCD150^{+}CD48^{-} (A) cells. Gating strategy is shown in 1 representative FACS blot per genotype (left). Summary of statistical analyses of individual LSK subpopulations (right; n = 4 per genotype, *$P < .05$). (D) Cell-cycle distributions of fraction A cells were determined by a combined DAPI and Ki-67 staining. One representative FACS blot per genotype is shown (left). Summary of statistical analyses (right; n = 4 per genotype, **$P < .01$, ***$P < .001$). (E) *Cdk6*$^{+/+}$ and *Cdk6*$^{-/-}$ mice were analyzed for lineage recovery (Gr1^{+}Mac1^{+}, CD19^{+}, and Ter119^{+} cells) 8 days after 5-FU treatment (n = 4 per genotype; *$P < .05$). i.p., intraperitoneally.

than in the control cohort (Figure 3E), but no differences in the numbers of apoptotic LSKs or fraction A cells were detectable (supplemental Figure 3B).

Reduced activation of dormant *Cdk6*$^{-/-}$ HSCs after poly(I:C) treatment

These findings indicate a possible malfunction of the most quiescent stem cells in fraction A of *Cdk6*$^{-/-}$ mice. To test this notion, we performed in vivo label-retaining experiments using 5-bromo-2′-deoxyuridine (BrdU). After a 70-day chase period, the most quiescent stem cell fraction remained BrdU-positive.[12,30] When we provoked HSC proliferation by injecting poly(I:C),[31] significantly more cells remained BrdU-positive in *Cdk6*$^{-/-}$ mice (Figure 4A), indicative of their restricted ability to leave quiescence. Experiments without prior BrdU labeling confirmed that significantly more cells of fraction A in *Cdk6*$^{-/-}$ mice remain in the G_0 phase (supplemental Figure 4A-B). No alterations in the cell-cycle distribution of fraction B or C cells were detected (supplemental Figure 4C).

Exit from quiescence is controlled by a network of transcriptional regulators including *Nurr1*, *Egr1*, *Runx1*, and *p21*^{CIP1}. Poly(I:C) treatment induced *Cdk6* expression by about 3-fold in the cells of fraction A, whereas the level of *Cdk4* messenger RNA (mRNA) remained unchanged (Figure 4B). There were no changes in the levels of other transcription factors implicated in the regulation of stem cell quiescence (*p16*^{INK4A}, *p53*, *p27*^{KIP1}, *p21*^{CIP1}, *Smad3*, *Smad4*, *Smad7*, *Foxo1a*, *JunB*, and *Runx1*; supplemental Figure 5A). Consistent with previous findings,[5] we detected altered *Notch1* regulation (supplemental Figure 5A). Furthermore, we observed increased *Nurr1* expression in fraction A cells of nonstimulated *Cdk6*$^{-/-}$ mice, but no differences between *Cdk6*$^{+/+}$ and *Cdk6*$^{-/-}$ mice in the levels of *Nurr1* RNA in stimulated cells of fraction A. The changes in expression of *Notch1*[32] and *Nurr1* are insufficient to account for the phenotype of *Cdk6*$^{-/-}$ HSCs. We found a pronounced downregulation

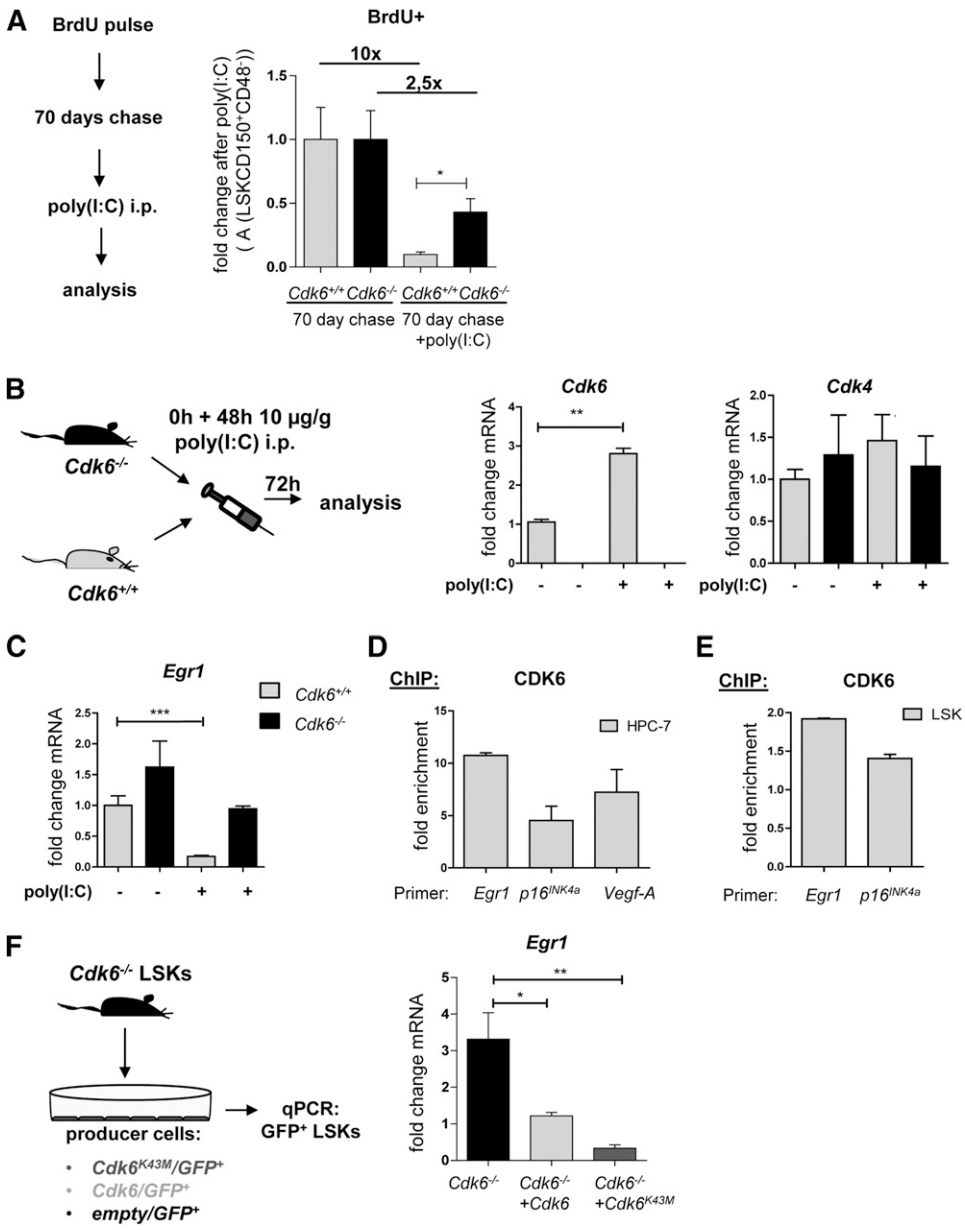


Figure 4. Impaired activation of dormant *Cdk6*$^{-/-}$ HSCs after poly(I:C) treatment. (A) *Cdk6*$^{+/+}$ and *Cdk6*$^{-/-}$ mice received a pulse of BrdU i.p. (1 mg/mouse) that was followed by a 10-day period of BrdU administration via drinking water (1 mg/mL) (n = 8 per genotype). After a 70-day chase period, the mice cohort was split and BrdU^{+} fraction A cells were analyzed with (n = 4 per genotype) or without (n = 4 per genotype) a 24-hour preceding injection of poly(I:C) (10 μg/g body weight). Treatment with poly(I:C) resulted in a 10-fold reduction of BrdU^{+} fraction A cells in *Cdk6*$^{+/+}$, but only in a 2.5-fold reduction in *Cdk6*$^{-/-}$ mice. (B) *Cdk6* and *Cdk4* mRNA expression levels in fraction A cells of *Cdk6*$^{+/+}$ and *Cdk6*$^{-/-}$ mice that had received poly(I:C) or PBS are shown (n = 3 for each genotype; qPCR analyses were performed in triplicate; $^{**}P < .01$). (C) *Egr1* mRNA expression levels in fraction A cells of *Cdk6*$^{+/+}$ and *Cdk6*$^{-/-}$ mice that had received poly(I:C) or PBS are shown (n = 6 for each genotype; qPCR analyses were performed in triplicate; $^{***}P < .001$). (D-E) ChIP assays were performed in an HPC-7 hematopoietic progenitor cell line (D) and in primary *Cdk6*$^{+/+}$ LSKs (E). Protein–DNA complexes were immunoprecipitated using antibodies directed against CDK6 and analyzed by qPCR for their presence on the *Egr1* promoter region. *Vegf-A* and/or *p16*^{INK4a} promoter regions were used as positive controls. Bar graphs depict fold enrichment over a negative region downstream of CD19. (F) *Cdk6*$^{-/-}$ LSKs were sorted and coincubated with *Cdk6*/GFP^{+}, *Cdk6*^{K43M}/GFP^{+}, or empty/GFP^{+} GP^{+} producer cells (n = 3 per genotype). After 48 hours, GFP^{+} cells were high-purity sorted by FACS and analyzed by qPCR. Bar graphs depict *Egr1* mRNA expression levels (technical triplicates; $^{*}P < .05$, $^{**}P < .01$).

of *Egr1* upon poly(I:C) treatment of fraction A cells of *Cdk6*$^{+/+}$ mice that was not observed in fraction A cells of *Cdk6*$^{-/-}$ mice (Figure 4C). Downregulation of *Egr1* is necessary for HSCs to exit quiescence.[13]

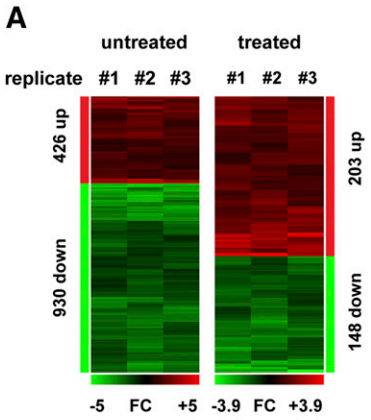
CDK6 directly regulates *Egr1* expression in hematopoietic cells

Poly(I:C) stimulation induces *Cdk6* and suppresses *Egr1*, suggesting that CDK6 may directly regulate *Egr1* expression. We have identified a transcriptional role for CDK6 in lymphoid malignancies[7] and so we used BCR-ABL^{p185+}–transformed pro-B cells for initial investigations of the *Egr1* promoter. *Egr1* is expressed at high levels in transformed differentiated cells and functions as a proto-oncogene in certain tumor types.[33-35] Consistently, we find high levels of *Cdk6* mRNA paralleled by a statistically significant upregulation of *Egr1* in transformed BCR-ABL^{p185+} cells (supplemental Figure 6A). Chromatin immunoprecipitation (ChIP) analysis revealed the presence of CDK6 at the *Egr1* promoter at levels comparable to its binding to the *p16*^{INK4A} and *Vegf-A* promoters, which are known transcriptional targets of CDK6 in this cell type (supplemental Figure 6B).[7] ChIP assays with the hematopoietic progenitor cell line HPC-7 and with sorted LSKs (with *Vegf-A* and/or *p16*^{INK4A} promoters as positive controls) showed that CDK6 also binds to the *Egr1* promoter in these cell types (Figure 4D-E).

CDK6 has recently been shown to influence transcription independently of its kinase function. To examine whether CDK6 activates *Egr1* in a kinase-dependent or kinase-independent manner, we used the kinase-dead mutant *Cdk6*^{K43M}. ChIP assays confirmed that CDK6^{K43M} binds to the *Egr1* promoter in BCR-ABL^{p185+} pro-B cells (supplemental Figure 6C). Expression of *Cdk6*^{K43M} significantly regulated *Egr1* levels in BCR-ABL^{p185+} pro-B cells (supplemental Figure 6D) and in *Cdk6*$^{-/-}$ LSKs transduced with *Cdk6*, *Cdk6*^{K43M}, or empty vector control (Figure 4F; supplemental Figure 6E).

CDK6 is part of a transcription factor network that regulates stem cell quiescence

To investigate the effects of poly(I:C) stimulation of *Cdk6*$^{+/+}$ and *Cdk6*$^{-/-}$ cells in fraction A, we performed transcriptional profiling



B

untreated		treated	
Transcription factor	adjusted p-value	Transcription factor	adjusted p-value
Pax2	2,09E-20	Mafb	3,07E-17
Nurr1	1,65E-13	Klf4	7,47E-14
FOXI1	1,25E-11	GABPA	2,66E-03
Arnt	1,06E-05	Egr1	2,66E-03
SPI1 (PU.1)	1,06E-05	NFIL3	2,66E-03
ELK1	6,99E-05	MEF2A	0,002661
USF1	6,99E-05	Arnt	0,008636
FEV	1,39E-04	TBP	0,009417
Mafb	0,0001424	NKX3-1	0,01533

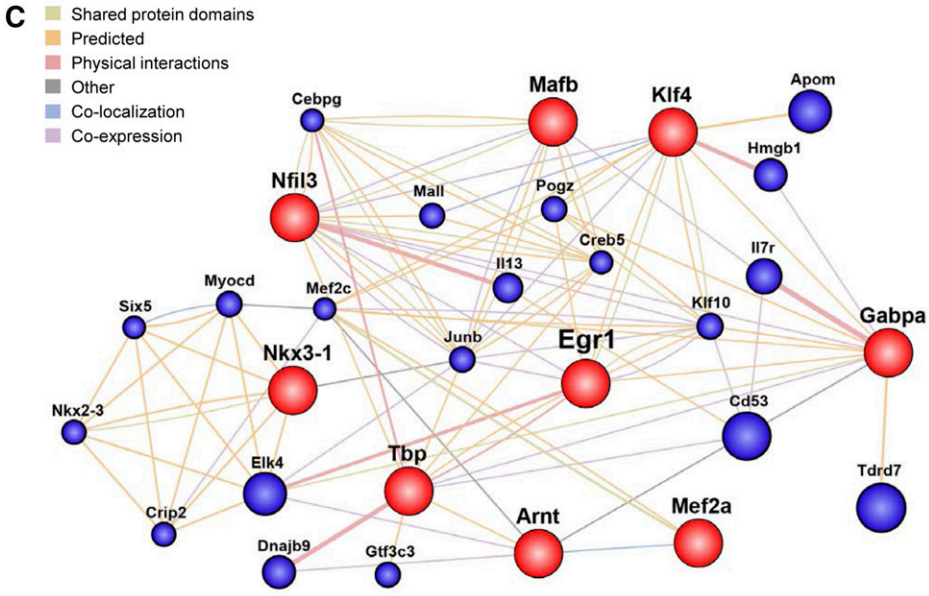


Figure 5. Transcriptional profiling of poly(I:C) treated and untreated *Cdk6*$^{-/-}$ HSCs. (A) A summary of transcripts up- (red) or down- (green) regulated (fold-change >2) in fraction A cells that were FACS-purified from either untreated or poly(I:C)-treated *Cdk6*$^{-/-}$ and *Cdk6*$^{+/+}$ mice (n = 3 per genotype). (B) Transcripts deregulated in either untreated or poly(I:C)-treated *Cdk6*$^{-/-}$ fraction A cells displayed promoter sequences significantly enriched in recognition sites for the indicated transcription factors compared with the respective *Cdk6*$^{+/+}$ controls. (C) GeneMANIA-computed association network of the transcription factors identified via their deregulated target gene programs in poly(I:C)-treated *Cdk6*$^{-/-}$ fraction A cells vs *Cdk6*$^{+/+}$ controls.

as described previously.[23] Untreated fraction A cells of *Cdk6*$^{-/-}$ mice had greater levels of 426 protein-coding transcripts and lower levels of 930 protein-coding transcripts compared with cells from *Cdk6*$^{+/+}$ mice. Upon poly(I:C) stimulation, differences between the genotypes were found in 203 (upregulated) and 148 (downregulated) protein-coding transcripts (Figure 5A, supplemental Tables 1 and 2). Gene ontology analysis suggested that the deregulated genes are involved in a variety of biological processes, as summarized in Table 1.

The majority of the known regulators of stem cell quiescence were not significantly affected, although there were significant changes to the levels of *Nurr1*, *Atm, Hoxc4*, and (especially) *Egr1* (Table 2). The outcome of a JASPAR-TFB screen for targets of specific transcription factors is summarized in Figure 5B. We found levels of *Nurr1* target genes (supplemental Table 3) to be significantly affected by the loss of CDK6 in unstimulated cells. In contrast, the key transcription factors with different activities in stimulated *Cdk6*$^{-/-}$ and *Cdk6*$^{+/+}$ cells included *Egr1* and *Klf4*. *Egr1* has been reported to regulate *Klf4*,[36] so we used GeneMANIA to investigate further interconnections between the individual regulators identified by JASPAR-TFB (Figure 5C). Array hit validation by quantitative polymerase chain reaction (qPCR) of individual genes found to be deregulated in untreated or poly(I:C)-treated cells of *Cdk6*$^{-/-}$ fraction A (*Klf4*, *PU.1*, *Arnt*) were consistent with our data set (supplemental Figure 7A). In summary, the findings indicate that the CDK6-*Egr1* axis represents an important part of a transcription factor network that controls exit from quiescence of HSCs.

The key role of CDK6 in regulating stem cell quiescence extends to LSCs

HSCs represent the LSCs' compartment in BCR-ABL^{p210+}–induced leukemia.[37] To investigate whether CDK6 regulates *Egr1* in LSCs, we infected *Cdk6*$^{+/+}$ and *Cdk6*$^{-/-}$ BM cells with a retrovirus encoding BCR-ABL^{p210}-IRES GFP and injected them into non-irradiated NSG mice (Figure 6A). This procedure triggers a CML-like disease that depends on the constant replenishment of peripheral leukemic cells by BCR-ABL^{p210+} LSCs. In line with published results,[37] all mice that received BCR-ABL^{p210+} *Cdk6*$^{+/+}$ BM succumbed to disease within 3 months, whereas only 1 of the 7 animals that received BCR-ABL^{p210+} *Cdk6*$^{-/-}$ cells became sick within this period (Figure 6B). Differences were even more explicit in a second round of transplantation, which again forced the BCR-ABL^{p210+} LSCs to repopulate. Although comparable amounts of BCR-ABL^{p210+} BM cells were used for transplantation, only mice that received *Cdk6*$^{+/+}$ leukemic cells succumbed to leukemia (Figure 6C). Mice that received BCR-ABL^{p210+} *Cdk6*$^{-/-}$ cells remained disease-free for up to 60 days. Analysis of the mice confirmed that *Cdk6*$^{-/-}$ LSCs

Table 1. Gene ontology analysis of transcripts deregulated in untreated and poly(I:C) treated *Cdk6*$^{-/-}$ fraction A cells

GO ID	Description	Representative genes upregulated	Representative genes downregulated
Untreated			
GO:0070887	Cellular response to chemical stimulus	Arnt, Cntf, Egr1, Ern1, Esr1, Etv5, Kif16b, Nr4a2, Rabgef1, Skil, Smad7	Aicda, Bcar1, Bmp2, Ccl6, Cxcr2, Lyst, Ngfr, Pax2, Tbx1, Timp2, Trib1
GO:0098552	Side of membrane	Amot, Cxcl9, Dlk1, Folr2,Hyal2, Il12rb1, Klre1	Cd74, Cd80, Ceacam2, Efna5, Fas, Fcer1a, Itga1, Rasa2
GO:0009605	Response to external stimulus	Bmp7, Chn1, Gbp6, Nr4a2, Rab38	Ablim1, Cxcr1, L1cam, Ngfr, Penk, Slit3
GO:0031012	Extracellular matrix	Chad, Col16a1, Col18a1, Dspp, Prss12	Ache, Gpc3, Leprel1, Lox, Lpl, Otog, Serac1, Vcan
GO:0048583	Regulation of response to stimulus	Amot, Egr1, Klre1, Rabgef1, Sybu	Acp5, Apod, Disc1, Fcgr3, Notch1, Slit3
GO:0009986	Cell surface	Cxcl9, Dlk1, Folr2, H2-K1, Klre1	Ache, Ceacam2, Clec7a, Efna5, Gfra1, Ramp1
GO:0043235	Receptor complex	Il12rb1, Rnmt	Cacng3, Card11, Egfr, Gpr160, Itga1
GO:0031224	Intrinsic component of membrane	Apool, B4galt7, Gpr125, Plscr4, Zdhhc20	Ache, Clcc1, Grina, Layn, Scn11a, Tmem160
GO:0032502	Developmental process	Catsper4, Dpf1, Nanog, Rbm19, Tshz1	Antxr1, Cdh4, Dkkl1, E2f7, Elk1, Tg
GO:0005615	Extracellular space	C1rl, Cntf, Eef1a1, Fam20c, Retn	Acta2, Cfb, Ctsk, Inhba, Lepr
GO:0040011	Locomotion	Bmp7, Hyal2, Nr4a1, P2ry1	Ablim1, Cemip, Dock4, L1cam, Snai2
Poly:IC treated			
GO:0070887	Cellular response to chemical stimulus	Ahr, Ctr9, Dgat2, Egr1, Egr2, Egr3, Klf4, Nfil3	Ackr4, Fam132a, Gcg, Phip, Pklr
GO:0001071	Nucleic acid binding transcription factor activity	Btg2, Fosl2, Hes1, Id3, Mafb	E2f7, Foxl2, Hmga1, Nkx2-2, Zfp628
GO:0016265	Death	Adamtsl4, Hcar2, Lgmn, Perp, Xaf1	Atm, Gcg, Optn, Phip, Wrn
GO:0010033	Response to organic substance	Dgat2, Egr1, Egr2, Egr3, Fosl2, Mgst1	Fgf8, Gpd1, Hpx, Iigp1, Pklr
GO:0003677	DNA binding	Csrnp1, Hes1, Hist1h1c, Nfil3, Tbx3	E2f7, Jrk, Sox6, Zfp518a, Zfp628

Most significantly deregulated functional pathways and their Gene Ontology (GO) codes are shown first.

had homed efficiently to the BM. Despite their presence, no signs of leukemia could be detected in the recipient animals (Figure 6D-E). BCR-ABL^{p210+} LSCs derived from *Cdk6*$^{+/+}$ and *Cdk6*$^{-/-}$ mice express identical surface markers, which are reminiscent of those on fraction C cells (supplemental Figure 8A). When we analyzed the cell cycle of the remaining *Cdk6*$^{+/+}$ and *Cdk6*$^{-/-}$ BCR-ABL^{p210+} LSKs, we again found more *Cdk6*$^{-/-}$ BCR-ABL^{p210+} LSKs in the G_0 phase (supplemental Figure 8B).

Table 2. Regulators of HSC quiescence

Gene symbol	Description	Fold-change untreated	*P* value untreated	Regulation untreated	Fold-change poly:IC	*P* value poly:IC	Regulation poly:IC
Egr1	Early growth response 1	2.90	2.11E-03	Up	2.62	1.25E-02	Up
Atm	Ataxia telangiectasia mutated homolog (human)	1.50	6.02E-01	—	2.08	4.62E-02	Down
Nurr1	Nuclear receptor subfamily 4, group A, member 2, transcript variant 1	4.03	1.01E-03	Up	2.68	7.45E-02	—
Cdkn2c	CDK inhibitor 2C (p18, inhibits CDK4)	2.21	2.36E-01	—	2.16	1.71E-01	—
Gfi1	Growth factor independent 1	1.12	6.56E-01	—	1.96	2.77E-02	—
Stat1	Signal transducer and activator of transcription 1, transcript variant 2	1.07	6.48E-01	—	1.75	2.24E-02	—
Hoxb9	Homeobox B9	1.27	4.77E-01	—	1.70	4,52E-02	—
Junb	Jun B proto-oncogene	1.21	2.92E-01	—	1.65	8,99E-02	—
Pml	Promyelocytic leukemia, transcript variant 2	1.22	8.64E-02	—	1.64	4.62E-02	—
Foxo4	Forkhead box O4	1.75	1.89E-03	—	1.54	2.93E-01	—
Hoxa4	Homeobox A4	1.03	8.27E-01	—	1.47	3.07E-01	—
Bmi1	Bmi1 polycomb ring finger oncogene	1.21	1.38E-01	—	1.38	2.76E-01	—
Trp53	Transformation related protein 53	1.52	1.81E-02	—	1.35	1.26E-01	—
Txnip	Thioredoxin interacting protein	1.75	3.40E-02	—	1.27	2.42E-01	—
Shh	Sonic hedgehog	1.25	2.30E-02	—	1.26	2.44E-01	—
Tal1	T-cell acute lymphocytic leukemia 1, transcript variant 2	1.07	6.91E-01	—	1.25	1.93E-01	—
Satb1	Special AT-rich sequence binding protein 1, transcript variant 2	1.65	1.71E-01	—	1.22	4.03E-01	—
Hoxc4	Homeobox C4	6.78	8.87E-03	Up	1.19	7.98E-01	—
Itch	Itchy, E3 ubiquitin protein ligase, transcript variant 2	1.21	2.00E-01	—	1.14	7.27E-01	—
Pbx1	Pre B-cell leukemia homeobox 1 (Pbx1), transcript variant a	1.27	7.09E-02	—	1.12	3.57E-01	—

Summary of HSC quiescence regulating genes that are differently regulated in *Cdk6*$^{-/-}$ fraction A cells (listed in order of "fold-change") in steady state ("untreated") or upon in vivo poly(I:C) stimulation. In the absence of CDK6, *Egr1* levels are significantly higher compared with control. This difference is even more prominent in the poly(I:C)-treated fraction, in which *Egr1* represents the most deregulated factor. Statistical significance (fold-change >2; $P < .05$).

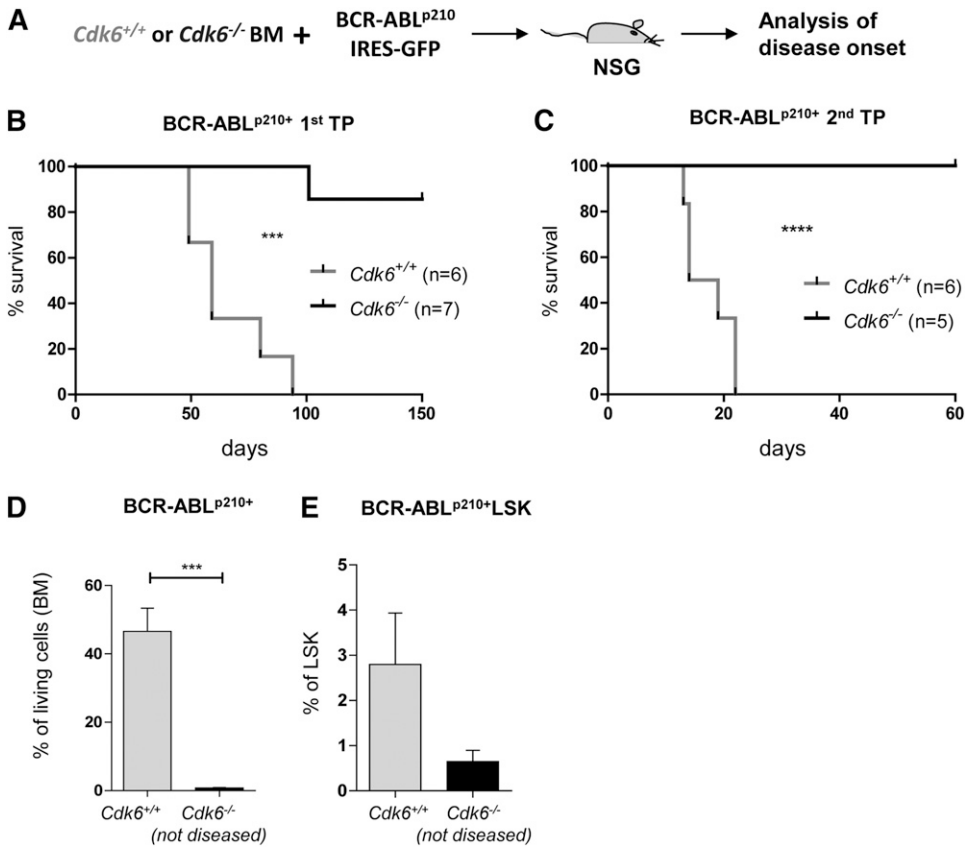


Figure 6. CDK6 is required for leukemia formation in vivo. (A) Experimental setup: *Cdk6*$^{+/+}$ and *Cdk6*$^{-/-}$ BM cells were co-cultivated on BCR-ABL^{p210} producer cells and 2 × 10^6 cells were injected i.v. in non-irradiated NSG mice. (B) Kaplan-Meier plot depicting disease onset of NSGs injected with *Cdk6*$^{+/+}$ or *Cdk6*$^{-/-}$ BCR-ABL^{p210+} leukemic cells (n = 6 and n = 7, respectively). Only 1 of 7 mice injected with *Cdk6*$^{-/-}$ BCR-ABL^{p210+} cells became diseased. All mice injected with *Cdk6*$^{+/+}$ BCR-ABL^{p210+} cells became diseased within 3 months (***$P < .001$). (C) 2 × 10^6 BM cells of diseased animals were transplanted in a second transplantation round and disease onset was monitored. None of the mice injected with *Cdk6*$^{-/-}$ BCR-ABL^{p210+} cells became diseased, but all mice injected with *Cdk6*$^{+/+}$ BCR-ABL^{p210+} cells became diseased rapidly within 3 weeks (n = 5 and n = 6, respectively; ****$P < .0001$). (D) The experiment (Figure 6C) was terminated after 60 days and *Cdk6*$^{-/-}$ nondiseased animals were euthanized. Contribution of *Cdk6*$^{-/-}$ BCR-ABL^{p210+}–transformed cells (BM) was compared with diseased control animals at the time of terminal disease (n = 3 per genotype, ***$P < .001$). (E) Frequencies of BM BCR-ABL^{p210+} LSKs of *Cdk6*$^{-/-}$ animals compared with *Cdk6*$^{+/+}$ (diseased) animals. *Cdk6*$^{-/-}$ BCR-ABL^{p210+} LSKs were detectable in the BM (n = 3 per genotype).

Egr1 knock-down rescues colony formation in *Cdk6*$^{-/-}$ BCR-ABL^{p210+} LSCs

In vitro colony formation assay confirmed the in vivo observation. After 48 hours of coculture of BM cells on retroviral producers, BCR-ABL^{p210+} LSKs were sorted and either seeded at 3 different cell numbers in methylcellulose to assess their (re-)plating potential or used for *Egr1* qPCR (Figure 7A). Eight days after plating, we observed a profound reduction of *Cdk6*$^{-/-}$ leukemic clones, irrespective of the number of cells initially plated (Figure 7B-C). After each , all colonies were harvested to determine the number of BCR-ABL^{p210+} LSK cells. Whereas LSK numbers increased in BCR-ABL^{p210+} *Cdk6*$^{+/+}$ cells, the numbers of *Cdk6*$^{-/-}$ BCR-ABL^{p210+} LSKs remained stable (Figure 7D). These observations confirm the crucial role of CDK6 in BCR-ABL^{p210+} LSCs.

Egr1 levels were significantly higher in *Cdk6*$^{-/-}$ BCR-ABL^{p210+} LSKs than in *Cdk6*$^{+/+}$ BCR-ABL^{p210+} LSKs (Figure 7E). To assess the functional relevance, we performed short hairpin RNA–mediated knockdowns for *Egr1*, validating the constructs in a hematopoietic cell line (BCR-ABL^{p185+} pro B cells) (supplemental Figure 9A) before introducing them into *Cdk6*$^{-/-}$ BCR-ABL^{p210+} LSKs. Colony formation assays revealed a highly significant increase in growth factor–independent colony numbers (Figure 7F-G) and thus confirmed the importance of the CDK6/*Egr1* axis in BCR-ABL^{p210+}–mediated leukemogenesis.

Discussion

HSC homeostasis requires the precise regulation of cell proliferation because the maintenance of long-term repopulation capacity is crucial for the ability to produce blood cells. Dormant HSCs represent a reservoir for hematopoiesis and are ready to be rapidly activated when required.[16] We identified a dual role for CDK6 in HSCs—in addition to its function as a cell-cycle kinase, CDK6 downregulates *Egr1* by directly binding to its promoter. This important function is also performed in LSCs, because *Cdk6*$^{-/-}$ BCR-ABL^{p210+}-transformed LSKs are incapable of inducing disease.

We failed to detect any changes in LSK populations in young *Cdk6*$^{-/-}$ mice under steady-state conditions. This finding is consistent with the initial results of Hu et al,[5] although the group subsequently[38] reported slightly reduced numbers of LSKs in *Cdk6*$^{-/-}$ mice. It is conceivable that the minor differences reported in the second article arise as a result of the knockout strategy employed or from differences in housing conditions. We are not able to explain this discrepancy; our results are fully consistent with the earlier article from Hu et al.

CDK6 has a unique role under stress conditions when *d*-HSCs are forced to exit G_0. CDK6 is not required under steady-state conditions, when CDK4 alone is sufficient to drive proliferation. *Cdk6* is upregulated in HSCs under stress conditions, showing that it has an important role when a rapid and fast response of HSCs is required. *Cdk4* expression remains constant, and we failed to detect any compensatory upregulation in *Cdk6*$^{-/-}$ animals.

We have recently shown that CDK6 is not only a cell-cycle kinase but also a direct regulator of transcription.[7] Deleting CDK6 did not result in consistent differences in the levels of known cell-cycle regulators such as $p21^{Cdkn1a}$, $p27^{Cdkn1b}$, and $p16^{INK4a}$ or of in the transcription factors *p53*, *JunB*, *Runx1*, and members of the transforming growth factor-β (TGF-β) pathway. Hu et al have reported altered regulation of Notch target genes in *Cdk6*$^{-/-}$ T cells[5]; we have

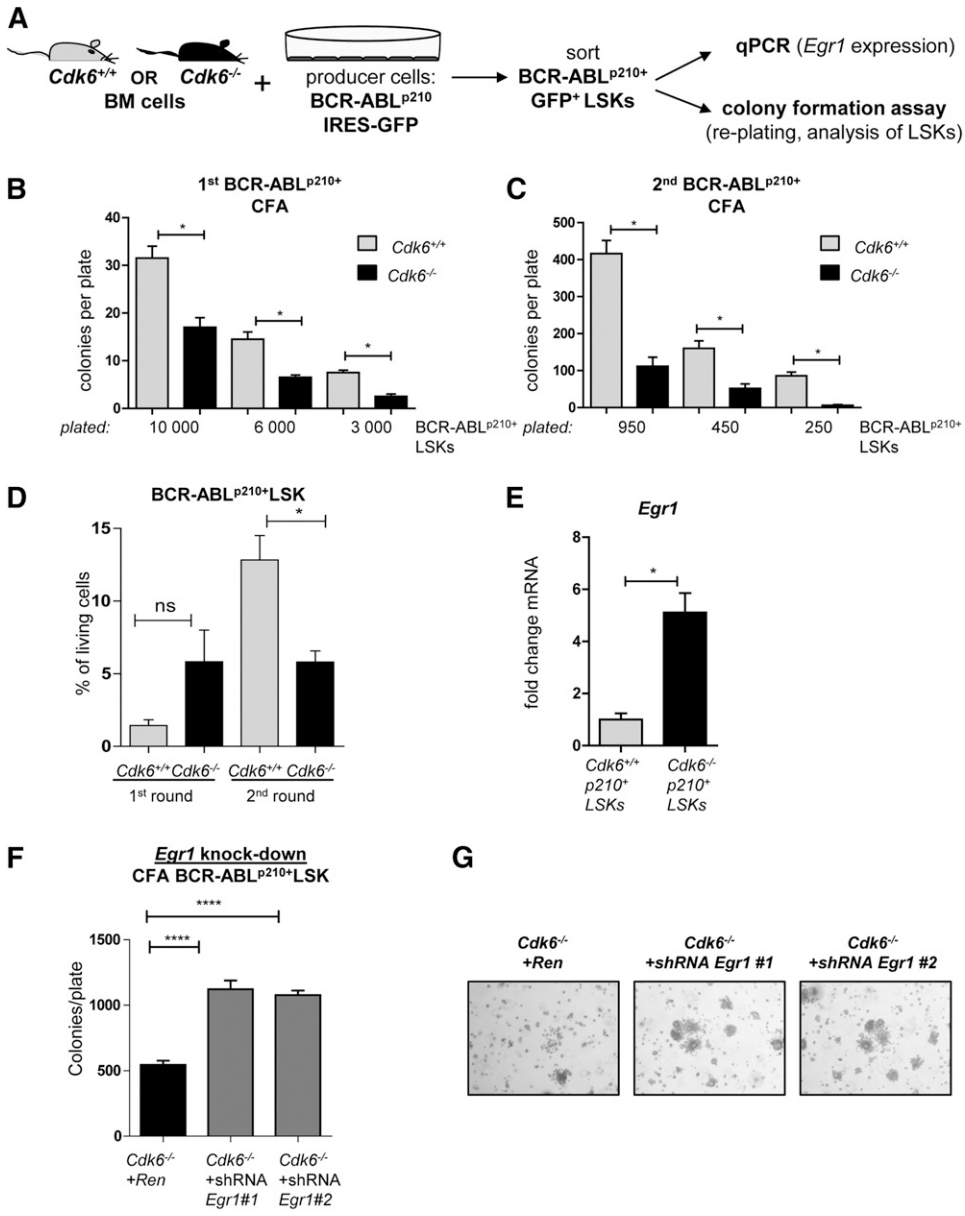


Figure 7. CDK6 influences (re-)plating capacities of BCR-ABL^{p210+} LSKs in vitro. (A) Experimental setup: *Cdk6*$^{+/+}$ and *Cdk6*$^{-/-}$ BM cells were cocultivated on BCR-ABL^{p210} producer cells for 48 hours, sorted by high-purity FACS, and either subjected to colony formation assays (CFA) (B-D) or analyzed by qPCR (E). (B) Three different cell numbers of BCR-ABL^{p210+} LSKs were seeded and colony numbers were counted 8 days after coculture (technical duplicates; *$P < .05$). (C) All colonies were harvested and reseeded to a second round of replating. Colonies were counted after 8 days (technical duplicates; *$P < .05$). (D) After each round, colonies were harvested and analyzed by FACS for the presence of remaining BCR-ABL^{p210+} LSKs (*$P < .05$). (E) BCR-ABL^{p210+} LSKs were sorted by FACS and *Egr1* expression was analyzed by qPCR (BM cells of 3 individual mice per genotype were pooled; qPCR was performed in technical triplicates; *$P < .05$). (F) Knockdown constructs *Egr1* #1 and *Egr1* #2 or a control vector targeting Renilla were introduced into *Cdk6*$^{-/-}$ BCR-ABL^{p210+} LSKs and subjected to colony formation. Colonies were again counted 8 days after seeding (****$P < .0001$). (G) Representative pictures of colonies on day 8 (magnification: ×4).

confirmed this finding in HSCs and show that the mRNA levels of *Notch1* depend on CDK6 both under steady-state conditions as well as upon poly(I:C) treatment. Moreover, we find deregulation of *Nurr1*, a nuclear receptor and transcription factor that is known to be involved in HSC quiescence. HSCs overexpressing *Nurr1* are capable of homing but fail to replenish the blood system.[39] Significant differences in *Nurr1* expression were only found in nonstimulated HSCs, where *Nurr1* was the dominant transcriptional regulator that distinguishes *Cdk6*$^{-/-}$ HSCs from controls. The difference is no longer as pronounced in stimulated HSCs, where downregulation of *Nurr1* occurs independently of CDK6. Effects on *Nurr1* thus do not correspond to the phenotype of CDK6 deletion, confirming that other mechanisms are involved.

All of our lines of investigation are consistent with the idea that the effects of CDK6 deletion are mediated to a large extent by the regulation of *Egr1*. In quiescent HSCs, *Egr1* is expressed at high levels and it must be downregulated to enable cells to proliferate upon stress.[13] CDK6 is part of the transcriptional apparatus that suppresses *Egr1*. CDK6 thus performs a dual function in HSCs: it allows them to exit quiescence in a kinase-independent manner, whereas its kinase-dependent role in the cell cycle is beyond doubt. The kinase-dead mutant CDK6^{K43M} regulates *Egr1* in the same manner as wild-type CDK6. EGR1 is an immediate-early transcription factor with functions in stress responses, growth control, and apoptosis.[40-42] Its pleiotropic functions are possible because it operates in a highly tissue-specific manner by interacting with various other transcriptional regulators.[40] Mice lacking *Egr1* have significantly more dividing HSCs, defining EGR1 as a central coordinator of stem cell homeostasis.[13]

Egr1 is frequently deregulated in transformed cells and in tumor tissue.[43,44] *Egr1* may function as a tumor suppressor in certain hematopoietic malignancies, including myeloid leukemia. Consistently, portions of chromosome 5 are frequently lost in myelodysplastic syndromes and AML, and *Egr1* is among the genes frequently affected.[45] Mice haploinsufficient for *Egr1* are prone to develop myeloid disorders upon treatment with *N*-ethyl-*N*-nitrosourea.[46,47]

Furthermore, *Egr1* is known to oppose the differentiation block inflicted by enforced c-Myc expression in myeloblastic leukemia.[48] The mechanisms underlying this effect are unclear, and *Egr1* has been postulated to interfere with p53.[49] It has also been proposed as an upstream regulator of various tumor suppressors including TGF-β1 and phosphatase and tensin homolog.[50] Whether and how the CDK6/EGR1 axis acts in other hematopoietic diseases driven by LSCs remains to be determined.

Maintaining the balance between proliferation and differentiation is not only important in recovery from hematopoietic stress, it is also a central issue in leukemia patients.[22,51] HSCs play a crucial part in malignancies such as CML, which is reflected by the fact that only the transplantation of transformed HSCs is able to induce murine BCR-ABL^{p210+}–driven myeloid leukemia. In CML, quiescent LSCs represent the leukemic stem cell compartment and are protected from conventional chemotherapy and tyrosine kinase inhibitors, allowing them to survive for many years and posing a significant challenge to therapeutic attempts.[52,53] Imatinib represents a major breakthrough in therapy, although it only targets the peripheral symptoms of leukemia. The idea of inhibiting CDK6 may seem provocative, but our data show that it warrants further investigation. It may be beneficial to develop inhibitors that specifically target CDK6's ability to regulate transcription. CDK4 and CDK6 are clearly nonredundant with regard to arousing *d*-HSCs and LSCs. LSCs may be more dependent on CDK6 than their nontransformed counterparts under steady-state conditions, and the difference may represent a possibility to distinguish and eliminate LSCs. Because the requirement for CDK6 is restricted to conditions of stress, including oncogenic stress, CDK6 inhibitors would not affect hematopoiesis under steady-state conditions and so might have fewer side effects than currently available forms of leukemia treatment.

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Authorship

Contribution: V.S. was the principal investigator and takes primary responsibility for the article; R.S., A.H., F.B., K.K., C.S., A.T., M.P.M., M.S.R., and G.H. performed the laboratory work for this study; R.S., A.H., and V.S. wrote the manuscript; M.M., K.K., and J.Z. contributed to development of methodology; and R.S., A.H., F.B., S.Z.M., M.P.M., K.K., and M.M. were helpful in interpretation of the data.

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