Intrinsic and extrinsic regulators of stem cell function in normal and malignant hematopoiesis

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CHAPTER 5 //

DEPLETION OF SAM50 SPECIFICALLY TARGETS BCR-ABL-EXPRESSING LEUKEMIC STEM AND PROGENITOR CELLS BY INTERFERING WITH MITOCHONDRIAL FUNCTIONS

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(under revision in Stem Cells and Development)

ABSTRACT

A high proliferation rate of malignant cells requires an increased energy production, both by anaerobic glucose metabolism and mitochondrial respiration. Moreover, increased levels of mitochondria-produced reactive oxygen species (ROS) promote survival of transformed cells and contribute to the disease progression both in solid tumors and leukemia. Consequently, interfering with mitochondrial metabolism has been used as a strategy to specifically target leukemic cells. SAM50 is a mitochondrial outer membrane protein involved in the formation of mitochondrial intermembrane space bridging complex (MIB). Although the importance of SAM50 in maintaining MIB integrity and in the assembly of mitochondrial respiratory chain complexes (MRC) has been described, its specific role in the normal and leukemic hematopoietic cells remains unknown. We observed that human leukemic cells display a specific dependence on SAM50 expression, as downregulation of SAM50 in BCR-ABL-expressing, but not normal human hematopoietic stem and progenitor cells (HSPCs) caused a dramatic decrease in growth, colony formation and replating capacity. Mitochondrial functions of BCR-ABL-expressing HSPCs were compromised, as seen by a decreased mitochondrial membrane potential and respiration. This effect of SAM50 downregulation was recapitulated in normal HSPCs exposed to cytokine-rich culture conditions that strongly enhanced proliferation. Both oncogene-transduced and cytokine-stimulated HSPCs showed increased mitochondrial membrane potential and increased ROS levels compared to their normal counterparts. Therefore, we postulate that human leukemic HSPCs are highly dependent on the proper functioning of mitochondria and that disruption of mitochondrial integrity may aid in targeting leukemic cells.

INTRODUCTION

Metabolic alterations are one of the hallmarks of carcinogenesis. An increased division rate of malignant cells results in elevated energy requirements, and consequently a more active metabolism. The phenomenon known as the Warburg effect describes the propensity of cancer cells to produce lactic acid from glucose even in aerobic conditions [1]. In fact, malignant cells utilize the anaerobic pathway of glucose metabolism next to mitochondrial respiration, which results in an increased glucose consumption rate and increased ATP production [2,3]. As a byproduct of their increased metabolism tumor cells generate high levels of reactive oxygen species (ROS), with mitochondria being one of the main sources [4–7]. Although elevated ROS levels are detrimental for healthy cells, in tumor cells they induce a variety of signaling pathways and activate transcription factors that promote growth, survival and malignant behavior [8–17]. Moreover, cancer cells counteract the overproduction of ROS by upregulating the antioxidant systems to prevent ROS-induced macromolecule damage, senescence and apoptosis induction [18–21].

The important role of mitochondrial metabolism for energy production and redox homeostasis has also been described in hematological malignancies. In acute myeloid leukemia (AML), inhibition of mitochondrial translation disrupted expression of mitochondrial respiratory complex (MRC) proteins and decreased respiration, specifically targeting leukemic cells [22]. In CML, deregulated expression of MRC components has been observed in leukemic stem cells (LSCs), indicating an increased level of oxidative phosphorylation that could reflect their high proliferation rate [23]. Elevated ROS levels have also been described in several leukemia models. Leukemic cells expressing internal tandem duplication of FLT3 (FLT3-ITD), the JAK2(V617F) activating mutation or the BCR-ABL kinase have shown increased levels of ROS causing DNA double strand breaks (DSB) to occur. This in turn has resulted in genomic instability leading to leukemia progression and development of therapy-resistant clones [24–27]. Moreover, elevated ROS levels increased survival of BCR-ABL-expressing cells through the activation of the PI3K/Akt signaling pathway [28]. Recently, RAC2 has been identified as a new key player in maintaining mitochondrial function in leukemic cells. In BCR-ABL-expressing cells, RAC2 has been shown to alter mitochondrial membrane potential and electron flow through the MRC complex III, generating high levels of ROS and causing DNA damage [29]. Downregulation of RAC2 in human BCR-ABL-transformed cord blood (CB) cells and primary blast crisis CML (BC CML) cells resulted in decreased mitochondrial membrane potential and decreased ROS production. Consequently, RAC2-deficient primitive leukemic cells showed increased apoptosis and diminished proliferation and self-renewal (Capala et al, submitted). We have identified that RAC2 may be required for the proper functioning of mitochondria due to its direct interaction with mitochondrial transport proteins, such as SAM50 and Metaxin 1 (Capala et al, submitted). These proteins, together with Metaxin 2, constitute the sorting and assembly machinery (SAM) in the mitochondrial outer membrane [30–32]. The SAM complex, by interacting with the mitochondrial inner membrane proteins mitofilin and CHCHD3

forms the mitochondrial intermembrane space bridging (MIB) complex involved in the assembly of MRC complexes. Consequently, downregulation of SAM50 was shown to destabilize the MIB complex, causing defective assembly of respiratory complexes and structurally abnormal cristae [33]. The specific role of SAM50 in normal and leukemic hematopoietic cells, however, remains unknown.

Here we report that human leukemic cells are specifically dependent on SAM50 expression. Downregulation of SAM50 in BCR-ABL-expressing, but not normal human hematopoietic stem and progenitor cells (HSPCs) cause a dramatic decrease in growth, colony formation and replating capacity, as well as a decreased mitochondrial membrane potential and respiration. This effect of SAM50 downregulation can be recapitulated in normal HSPCs when exposed to cytokine-rich culture conditions that strongly enhance proliferation. Both oncogene-transduced and cytokine-stimulated HSPCs show increased mitochondrial membrane potential and increased ROS levels compared to their normal counterparts. Therefore, we postulate that human leukemic HSPCs are highly dependent on the proper functioning of mitochondria and that disruption of mitochondrial integrity may aid in targeting leukemic cells.

MATERIALS AND METHODS

PRIMARY CELL ISOLATION AND CULTURE CONDITIONS

Neonatal cord blood (CB) was obtained from healthy full-term pregnancies after informed consent in accordance with the Declaration of Helsinki from the obstetrics departments of the University Medical Centre Groningen (UMCG) and Martini Hospital Groningen, Groningen, The Netherlands. All protocols were approved by the Medical Ethical Committee of the UMCG. After separation of mononuclear cells with lymphocyte separation medium (PAA Laboratories, Coble, Germany), $CD34^+$ cells were isolated using a magnetically activated cell sorting (MACS) CD34 progenitor kit (Miltenyi Biotech, Amsterdam, The Netherlands). For the MS5 co-culture experiments, cells were grown in Gartner's medium consisting of α-modified essential medium (α–MEM; Fisher Scientific Europe, Emergo, The Netherlands) supplemented with 12.5% heat-inactivated fetal calf serum (Lonza, Leusden, The Netherlands), 12.5% heat-inactivated horse serum (Invitrogen, Breda, The Netherlands), 1% penicillin and streptomycin, 2 mM glutamine (all from PAA Laboratories), 57.2 μM β-mercaptoethanol (Merck Sharp & Dohme BV, Haarlem, The Netherlands) and 1 μM hydrocortisone (Sigma-Aldrich Chemie B.V., Zwijndrecht, The Netherlands). Alternatively, co-cultures were expanded in Gartner's medium supplemented with 20 ng/mL interleukin 3 (IL-3; Gist-Brocades, Delft, The Netherlands) and stem cell factor (SCF; Amgen, Thousand Oaks, USA).

CELL LINES AND CULTURE CONDITIONS

293T embryonic kidney cells (ACC-635 DSMZ, Braunschweig, Germany) and PG13 packaging cells (ATCC CRL-10686, Wesel, Germany) were grown in DMEM medium with 200 mM glutamine (BioWhittaker, Veries, Belgium) supplemented with 10% FSC and 1% penicillin and streptomycin. K562 myelogenous leukemia cells (ACC-10,

DSMZ) were grown in RPMI medium with 200 mM glutamine (BioWhittaker) supplemented with 10% FCS, and 1% penicillin and streptomycin. MS5 murine stromal cells (ACC-441, DSMZ) were grown in αMEM with 200 mM glutamine (BioWhittaker) supplemented with 10% FCS and 1% penicillin and streptomycin.

RETRO- AND LENTIVIRUS GENERATION AND TRANSDUCTION

Stable PG13 producer cell lines of BCR-ABL retroviral constructs were generated and used as published previously [34]. Supernatants from the PG13 cells were harvested after 8 to 12 hours of incubation in human progenitor growth medium (HPGM; Cambrex, Verviers, Belgium) before the retroviral transduction rounds and passed through 0.45-µm filters (Sigma-Aldrich). Before the first transduction round, $CD34^+$ CB cells were pre-stimulated for 48 hours in HPGM supplemented with 100 ng/mL SCF, FLT3 Ligand (Flt3L; both from Amgen) and thrombopoietin (TPO; Kirin, Tokyo, Japan). Three rounds of transduction were performed on retronectin-coated 24-well plates in the presence of the same cytokines as for pre-stimulation and 4 µg/mL polybrene (Sigma-Aldrich). During the last round of retroviral transduction with BCR-ABL, lentiviral shRNA particles were also added as described below. Short hairpin RNA (shRNA) sequences targeting SAM50 were ligated into pHR'trip vector using AcsI and SbfI restriction sites as previously described (ref. Capala et al. in press). For the control, scrambled (SCR) shRNA sequence was used. 293T embryonic kidney cells were transfected using FuGENE6 (Roche, Almere, The Netherlands) with 3 µg pCMV Δ8.91, 0.7 µg VSV-G, and 3 µg of pHR'trip vector constructs (shSCR or shSAM50). After 24 hours medium was changed to HPGM and after 12 hours supernatant containing lentiviral particles was harvested and either stored at -80°C or used fresh for transduction of target cells. $CD34^+$ CB cells were subjected to three rounds of transduction with lentiviral particles, and BCR-ABL-transduced $CD34^+$ CB cells were subjected to 1 round of transduction with lentiviral particles, together with the last round of retroviral transduction, in the presence of prestimulation cytokines and 4 µg/mL polybrene (Sigma) on retronectin-coated 24-well plates (Takara, Tokyo, Japan). K562 cells were subjected to one round of transduction with lentiviral particles in presence of 4 µg/mL polybrene, on retronectin-coated 24-well plate. After transduction, transduced green fluorescent protein (GFP)-positive, truncated nerve growth factor receptor (NGFR)-positive or double-positive cells were sorted on a MoFlo sorter (Dako Cytomation, Carpinteria, CA, USA).

LONG-TERM CULTURES ON STROMA, CFC AND LTC-IC ASSAY

After sorting, 5×10^4 CB cells or 5×10^3 BCR-ABL cells were plated onto T25 flask pre-coated with MS5 stromal cells in 5 mL of Gartner's medium in duplicate. Co-cultures were kept at 37°C and 5% CO_2 and cells were demi-depopulated weekly for analysis. Images of cobblestones were acquired on Leica DMIL inverted phase microscope (Leica Microsystems, Eindhoven,

Netherlands). CFC assays were performed as previously described [35]. For the LTC-IC assay, CB cells ere plated in the range of 6 to 1458 cells per well in a 96-well plate using Gartner's medium. Methylcellulose (StemCell Technologies, Grenoble, France) supplemented with 20 ng/mL of IL-3, 20 ng/mL of interleukin-6 (IL-6; Gist-Brocades), 20 ng/mL of G-CSF, 20 ng/mL of c-kit ligand (Amgen), and 6 U/mL of erythropoietin (Epo; Cilag, Eprex, Brussels, Belgium) was added at week 5. Two weeks later, wells containing CFCs were scored as positive and the LTC-IC frequency was calculated using L-Calc software (StemCell Technologies). May-Grünwald Giemsa staining was used to stain cytospins. Cytospin preparations were evaluated and photographed using a Leica DM3000 microscope equipped with a Leica DFC420C digital camera at a total magnification of x400.

FLOW CYTOMETRY ANALYSIS AND SORTING

All fluorescence-activated cell sorter (FACS) analyses were performed on a FACScalibur (Becton-Dickinson [BD], Alpen a/d Rijn, the Netherlands) and data were analyzed using WinList 3D (Verity Software House, Topsham, USA). Cells were sorted on a MoFlo sorter. Antibodies: NGFR-APC, CD14-PE and CD15-APC were obtained from BD.

MEASUREMENT OF MITOCHONDRIAL FUNCTIONS

Mitochondrial membrane potential was measured by flow cytometry using hexamethylindodicarbocyanine iodide (DilC1; Life Technologies, Bleiswijk, The Netherlands) as described previously [36]. Briefly, 5×10^5 cells were incubated with 50 ng/mL DilC1 for 30 min at 37°C, washed twice in PBS, and DilC1 fluorescence was analyzed by FACS. To measure intracellular ROS levels, staining with CellROX Deep Red reagent was performed according to manufacturer's protocols (Life Technologies). Fluorescence of the probe was analyzed by FACS. OCR and ECAR were measured on XF^{e}24 Extracellular Flux Analyzer according to manufacturer's instructions (Seahorse Bioscience, Billerica, MA, USA). Briefly, XF^{e}24 Cell Culture Microplate was coated with poly-L-lysine (Sigma-Aldrich) and 2.5×10^5 K562 or BCR-ABL CB cells were plated per well in 100 µL XF Assay Medium (Seahorse Bioscience) supplemented with 4.5 g/L glucose 1 mM sodium pyruvate. Cells were allowed to attach for 30 min and XF Assay Medium was added to the final volume of 700 µL per well. XF^{e}24 Cell Culture Microplate was then loaded into XF^{e}24 Extracellular Flux Analyzer. OCR and ECAR values were derived as a mean of 5 consecutive measurements.

IMMUNOBLOTTING

Western blot analysis was performed according to standard protocols. Antibody against SAM50 and was kindly provided by Dr. V. Kozjak-Pavlovic (University of Würzburg, Würzburg, Germany). Secondary antibody (goat-anti-rabbit-HRP) was purchased from Dako Cytomation and used in 1:3000 dilutions. Binding of antibodies was detected by chemiluminescence, according to the manufacturer's instructions (Roche Diagnostics).

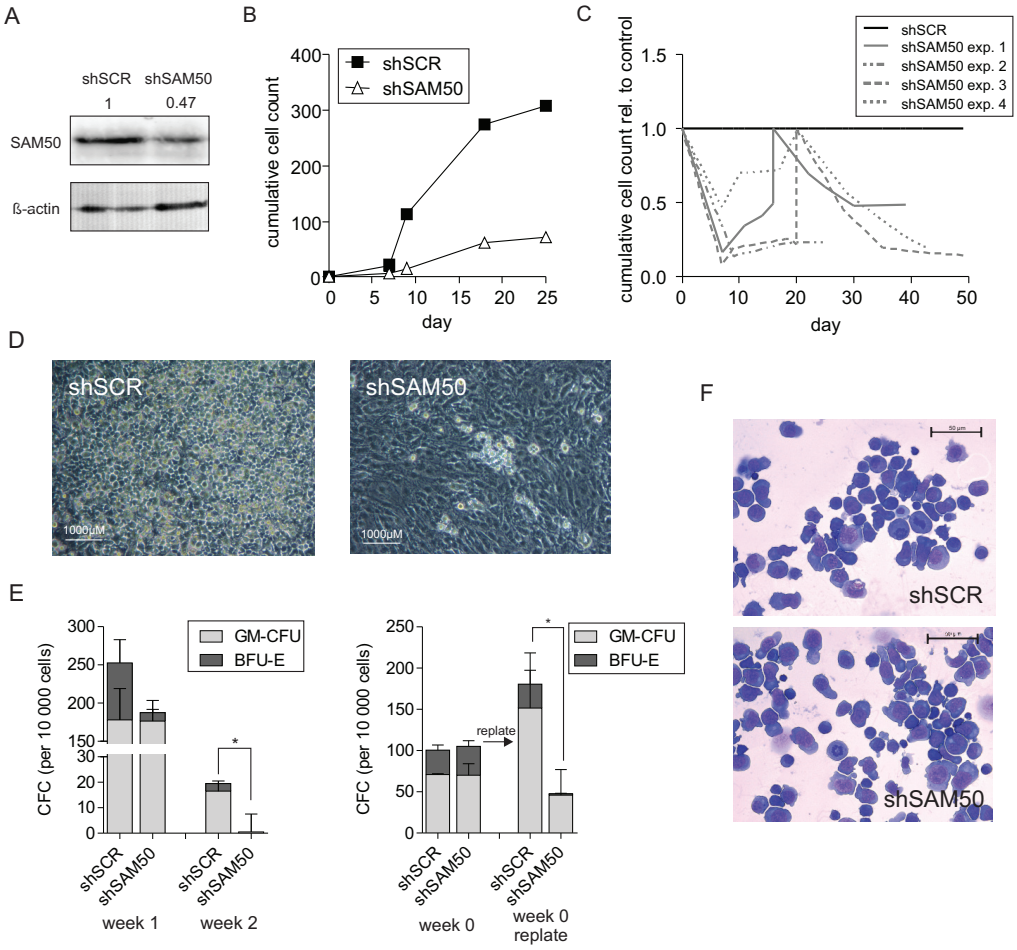


Figure 1. Human BCR-ABL-transduced HSPCs depend on SAM50 expression for their long-term expansion. (A) Cord blood (CB) CD34$^+$ stem/progenitor cells were co-transduced with a retroviral construct for BCR-ABL and either a control scrambled shRNA vector (shSCR) or with SAM50-targeting shRNA vectors (shSAM50). Transduced cells were sorted and used for Western blot analysis to determine SAM50 protein levels. Quantification of protein expression relative to control is indicated above each lane. (B) CB CD34$^+$ stem/progenitor cells were co-transduced with BCR-ABL and either control shSCR or with shSAM50. 5×10^3 double-transduced cells were sorted per group and plated on MS5 stromal cells. Cultures were demi-depopulated on indicated days for analysis and replated as indicated. The cumulative cell growth is shown for a representative experiment out of 4 independent experiments. (C) Fold reduction of cumulative cell growth of BCR-ABL and shRAC2-transduced CD34$^+$ CB cells in long-term co-culture as described in panel B, normalized to control cells. Cumulative expansion curves of 4 independent experiments are shown. (D) An example of day 14 cobblestones formed within the co-culture by shSCR- and shSAM50-transduced BCR-ABL cells. (E) Suspension cells from MS5 co-cultures as described in panel B were analyzed for progenitor frequency by CFC assay. 10^4 cells from each co-culture were plated in a CFC assay in methylcellulose in duplicate, and colonies were evaluated 2 weeks after plating. CFC cells were then harvested and 10^5 cells were re-plated to form secondary CFCs. Total CFC numbers are shown from a representative of 3 independent experiments. Error bars indicate standard deviation. (F) MGG-stained cytospins from week-1 suspension cells from co-cultures as described in panel B. CFU -GM, colony-forming unit-granulocyte-macrophage; BFU-E, burst forming unit-erythroid. * $P<0.05$.

STATISTICAL ANALYSIS

All values are expressed as means ± standard deviation (SD). Student's *t* test was used for all comparisons. Differences were considered statistically significant at $p<0.05$.

RESULTS

SAM50 EXPRESSION IS REQUIRED FOR THE LONG-TERM EXPANSION OF HUMAN BCR-ABL-EXPRESSING HSPCs

We have recently identified that the mitochondrial protein SAM50 is a *bona fide* interaction partner of RAC2 (Capala et al, submitted). To investigate in more detail the effect of SAM50 downregulation in human leukemic cells, we retrovirally transduced $CD34^+$ CB cells to overexpress the BCR-ABL oncogene. Simultaneously, lentiviral transduction with a shRNA-containing construct was performed to downregulate SAM50, and double-transduced cells were sorted and plated in stromal co-cultures. The efficiency of SAM50 knockdown was assessed by Western blot (Fig. 1A). Strikingly, SAM50-depleted cells reproducibly showed a profound growth impairment within the first weeks of co-culture and a largely reduced replating capacity (Fig. 1B, C). Moreover, cobblestone formation by the shSAM50-transduced cells was impaired, indicating that the most primitive cell fraction was affected by SAM50 downregulation (Fig. 1D). Although progenitor frequencies assessed by CFC assay directly after sorting and at week 1 of co-culture were not significantly lower in SAM50-depleted co-cultures, at week 2 they were markedly reduced. Importantly, also the replating potential of colony-forming cells was significantly decreased upon SAM50 downregulation (Fig. 1E). Differentiation of SAM50-depleted cells did not differ from the controls (Fig. 1F). Also cell viability, assessed by microscopical evaluation of the co-cultures and flow cytometry, was not affected by SAM50 downregulation (data not shown). Overall, these data suggested that BCR-ABL-transformed cells relied heavily on SAM50 expression for their long-term proliferation and self-renewal.

DEPLETION OF SAM50 RESULTS IN MITOCHONDRIAL DYSFUNCTION IN BCR-ABL-EXPRESSING HSPCs

Downregulation of SAM50 has been shown to destabilize the MIB complex, causing defective assembly of respiratory complexes [33]. To further investigate whether in a human leukemia model SAM50 depletion would result in mitochondrial impairment, we assessed the mitochondrial membrane potential in BCR-ABL-transduced $CD34^+$ CB cells upon knockdown of SAM50. FACS analysis using the DilC mitochondrial dye revealed that depletion of SAM50 caused a marked decrease in the mitochondrial membrane potential in BCR-ABL-transduced HSPCs, in accordance with our previous findings (Fig. 2A, Capala et al, submitted). However, the level of ROS measured in BCR-ABL HSPCs was not significantly altered upon SAM50 downregulation (Fig. 2B). To investigate whether mitochondrial oxidative phosphorylation was affected by SAM50 depletion we used the XF^e Analyzer of extracellular flux to measure the oxygen consumption

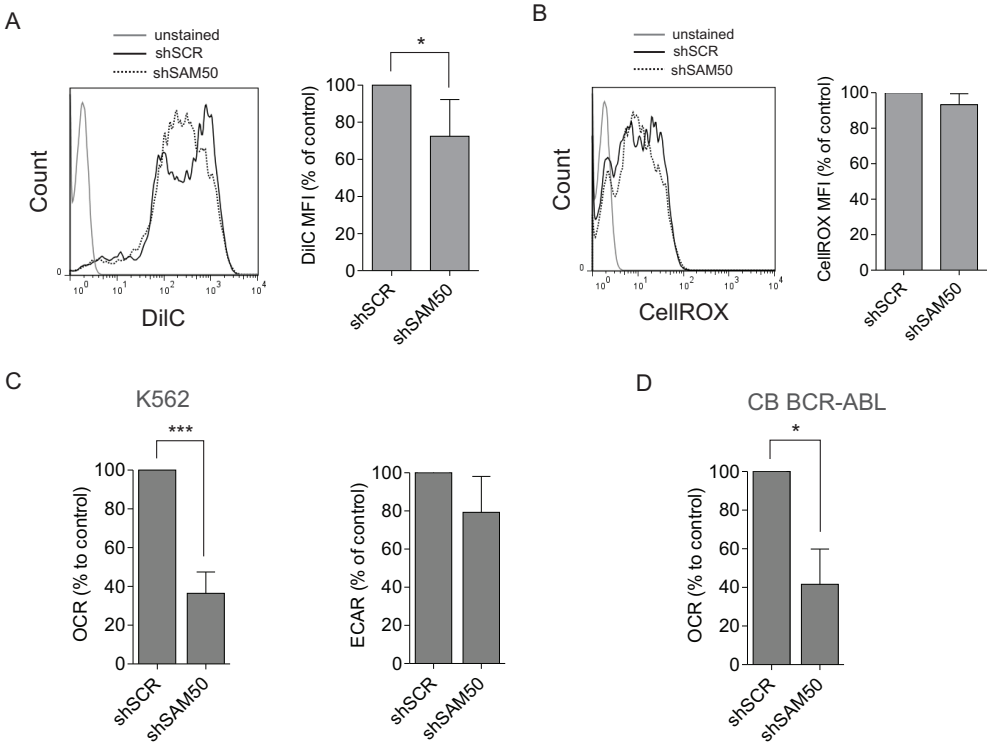


Figure 2. Depletion of SAM50 causes mitochondrial dysfunction in BCR-ABL-expressing HSPCs. (A) 5×10^3 CB $CD34^+$ stem/progenitor cells co-transduced with BCR-ABL and either control scrambled shRNA vector (shSCR) or with SAM50-targeting shRNA vectors (shSAM50) were sorted and plated on MS5 stromal cells. Suspension cells were harvested after 14 days of co-culture and stained with DilC to measure changes in mitochondrial membrane potential. Representative FACS plots and quantification of FACS measurements are shown, represented as changes in MFI relative to control. Average of 3 independent experiments is shown with standard deviation. (B) Suspension cells from the double-transduced co-cultures (BCR-ABL and shSCR or shSAM50) were harvested after 14 days and stained with CellROX to measure oxidative stress. Representative FACS plot and quantification of FACS measurements are shown, represented as changes in MFI relative to control. Average of 3 independent experiments is shown with standard deviation. (C) K562 cells were transduced with either shSCR or shSAM50 and analyzed by the XF^e Analyzer of extracellular flux. OCR and ECAR are represented as changes relative to control. Average of 3 independent experiments is shown with standard deviation. (D) Suspension cells from the double-transduced co-cultures (BCR-ABL and shSCR or shSAM50) as described in panel A were harvested after 14 days and analyzed by the XFe Analyzer of extracellular flux. OCR is represented as change relative to control. Average of 3 independent experiments is shown with standard deviation. OCR – oxygen consumption rate; ECAR - extracellular acidification rate. * $P<0.05$, *** $P<0.001$.

rate (OCR) and extracellular acidification rate (ECAR) of the BCR-ABL-expressing leukemic K562 cell line. OCR levels of shSAM50-transduced K562 cells were strongly reduced compared to control shSCR-transduced cells, indicative of decreased mitochondrial respiration. At the same time, ECAR levels were not significantly changed, indicating that glycolysis was not affected by SAM50 downregulation (Fig. 2C). Subsequently, we measured OCR in BCR-ABL and shSAM50 dou-

ble-transduced CD34+ CB cells. Also in this case, the OCR levels were significantly downregulated upon SAM50 depletion (Fig. 2D). Overall, these results showed that SAM50 downregulation negatively affected mitochondrial membrane potential and resulted in decreased mitochondrial respiration in BCR-ABL-expressing cells.

DOWNREGULATION OF SAM50 IN NORMAL HSPCs DOES NOT AFFECT PROLIFERATION, PROGENITOR AND STEM CELL FREQUENCIES OR MITOCHONDRIAL MEMBRANE POTENTIAL, BUT DECREASES REPLATING CAPACITY

Leukemic cells have been proven to be more sensitive to therapeutics targeting mitochondrial metabolism than normal hematopoietic cells [22]. Therefore, we investigated whether downregulation of SAM50 could also be used as a strategy to specifically target leukemic, but not normal hematopoietic cells. In order to study the long-term effects of SAM50 downregulation in human HSPCs, shRNA-transduced CD34+ CB cells were sorted and plated on a bone marrow stromal feeder layer for 5 weeks. The efficiency of SAM50 downregulation in shRNA-transduced cells was assessed by Western blot (Fig. 3A). During the co-culture, SAM50-depleted cells showed no proliferative disadvantage compared to control shSCR-transduced cells (Fig. 3B). Differentiation along the myeloid lineage was also unaffected (Fig. 3C). In addition, SAM50 downregulation did not result in a significant decrease of progenitor frequency, as assessed by CFC assay, or in the most primitive stem cell frequency in LTC-IC assay compared to control (1:302 vs. 1:224, p=0.4). Also, the mitochondrial functions as assessed by the measurement of the mitochondrial membrane potential and ROS levels were not different between SAM50-depleted and control HSPCs (Fig. 3F, G). However, the replating capacity of colony forming cells was strongly reduced upon SAM50 downregulation. This suggested that while under steady state conditions normal HSPCs were not affected by depletion of SAM50, normal HSPCs did heavily rely on SAM50 expression during stress conditions imposed on cells by serial replating of CFCs.

BCR-ABL-TRANSDUCED HUMAN HSPCs SHOW INCREASED MITOCHONDRIAL ACTIVITY

We speculated that the high sensitivity of BCR-ABL-expressing cells to SAM50 downregulation could originate from their increased mitochondrial activity. To this end, we directly compared mitochondrial membrane potential and ROS levels in normal and BCR-ABL-expressing human HSPCs. CD34+ cells isolated from a single batch of cord blood were used to eliminate inter-batch variability, and either co-transduced with BCR-ABL and shRNA constructs, or transduced with shRNA only. Sorted cells were plated in stromal co-cultures and FACS analyses were performed at the same time for normal and BCR-ABL-transduced cells. FACS analysis of DilC dye showed that the mitochondrial membrane potential was markedly increased in BCR-ABL-expressing HSPCs, compared to controls (Fig. 4A). ROS levels in BCR-ABL HSPCs were also increased, albeit to levels that were not statistically significant (Fig. 4B). Overall, these data

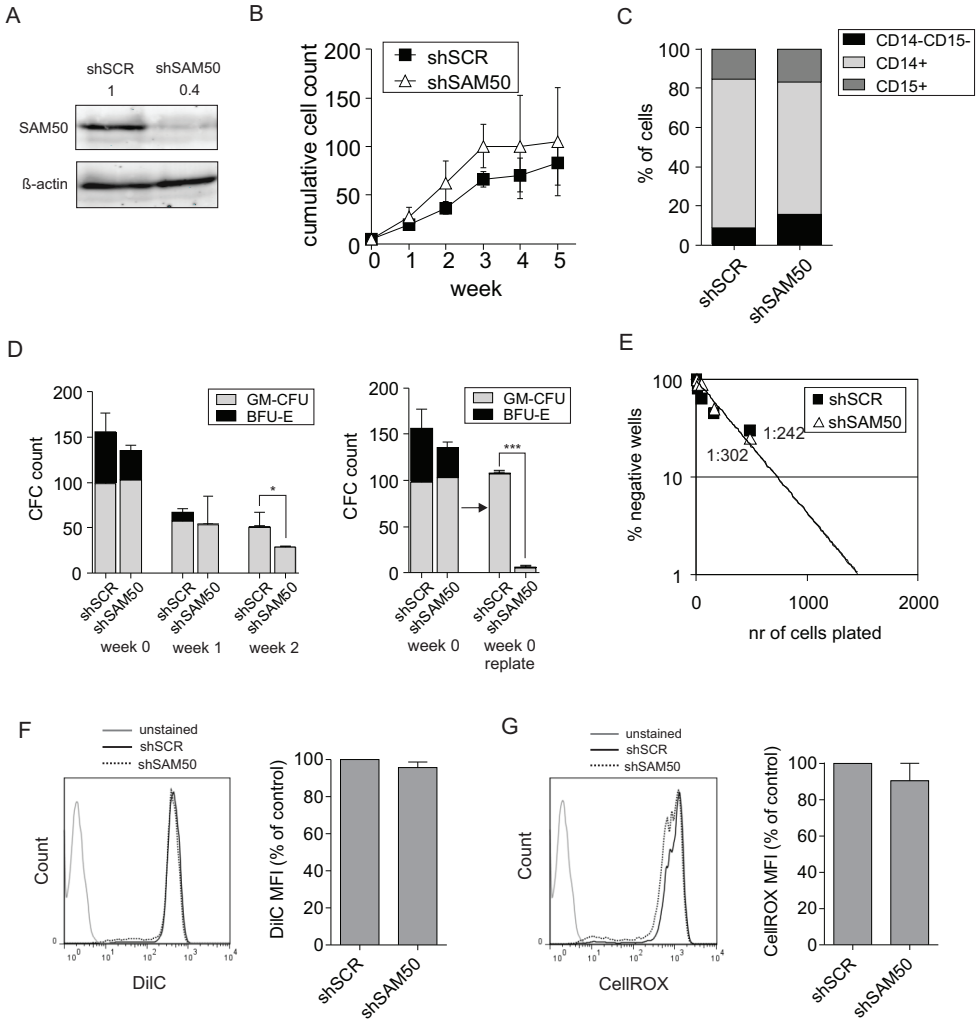
indicate that the mitochondrial activity of leukemic cells is altered, suggesting that this might explain their specific sensitivity to the disruption of mitochondrial function.

CYTOKINE-DRIVEN PROLIFERATION OF NORMAL HSPCs RESULTS IN INCREASED MITOCHONDRIAL ACTIVITY AND INCREASED SENSITIVITY TO SAM50 DOWNREGULATION

The BCR-ABL oncogene is a potent driver of proliferation of transformed CB cells [34,37]. Therefore, we wondered whether inducing a high proliferation rate on CB $CD34^+$ cells by culturing them in a cytokine-rich medium would increased their mitochondrial activity and render them more sensitive to SAM50 depletion. $CD34^+$ CB cells were cultured in a co-culture setting with or without the addition of cytokines (20 ng/mL stem cell factor and interleukin-3). As expected, cells cultured with cytokines showed a strongly increased proliferation (compare shSCR control data in Fig. 3B without cytokines with data in Fig. 5C with cytokines). FACS analysis using DilC staining showed a significant increase in mitochondrial membrane potential in cells cultured with the addition of cytokines (Fig. 5A). Also the ROS levels produced within these cells were increased, although the results did not reach statistical significance (Fig. 5B). Together, this showed that the cytokine-induced proliferation is paralleled by increased mitochondrial activity in CB cells. Next, we transduced $CD34^+$ CB cells with the SAM50-targeting or shSCR control shRNA construct, and transduced cells were sorted and plated on stroma in the same cytokine-rich medium. Strikingly, under the proliferation-driving culture conditions, shSAM50-transduced CB cells showed a marked proliferative disadvantage, which was not seen in the regular CB co-cultures without the extra addition of cytokines (Fig. 5B and 3B). Also the mitochondrial membrane potential was significantly decreased and ROS levels were slightly, but not significantly, reduced (Fig. 5D). Overall, these data suggested that high proliferation rate induced by cytokines increased the mitochondrial activity in CB cells, which in turn made them more sensitive to the disruption of mitochondrial functions by SAM50 downregulation.

DISCUSSION

Interfering with mitochondrial metabolism has previously been shown to be an effective strategy to specifically target leukemic cells. In AML cells, inhibition of mitochondrial translation with the drug tigecycline or by shRNA-mediated knockdown of the EF-Tu mitochondrial translation factor showed a selective anti-leukemic effect [22]. In chronic lymphocytic leukemia (CLL), interrupting the mitochondrial respiratory chain by a benzodiazepine derivative PK11195 specifically induced cell death in leukemic but not normal T and B-cells [38]. Also, in BCR-ABL-expressing leukemic cells pharmacological inhibition or decreased expression of small GTPase RAC2 selectively targeted leukemic and not normal HSPCs by decreasing their mitochondrial activity and ROS production [29,39,40] (Capala et al. submitted). In the current study we show that shRNA-mediated downregulation of a mitochondrial protein SAM50 resulted in a strongly reduced proliferation of leukemic but not



normal human HSPCs. The replating capacity of leukemic cells both in co-culture and CFC assay was strongly decreased, suggesting that also the more primitive, LSC-containing population was affected. Also the mitochondrial functions were affected by SAM50 downregulation in BCR-ABL expressing, but not normal CB cells.

SAM50, as a part of the MIB complex, is necessary for the assembly of the mitochondrial respiratory chain and formation of cristae [33]. In agreement with this, our experiments showed that downregulation of SAM50 resulted not only in a decreased mitochondrial membrane potential in BCR-ABL-expressing human HSPCs but also lower OCR, indicative of decreased oxidative phosphorylation. SAM50 expression was not increased in AML $CD34^+$ cells as compared to normal bone marrow (NBM) $CD34^+$ cells (unpublished observation) and therefore could not account for the dependence of leukemic cells on SAM50. When we directly compared normal and BCR-ABL-transduced CB $CD34^+$ cells, we observed an increased mitochondrial

Figure 3. SAM50 downregulation affects replating capacity, but not proliferation or progenitor and stem cell frequencies of human HSPCs in stromal co-cultures. (A) Cord blood (CB) $CD34^+$ stem/progenitor cells were transduced with control scrambled shRNA vector (shSCR) or with SAM50-targeting shRNA vectors (shSAM50), sorted and used for RNA extraction. Transduced cells were sorted and used for Western blot analysis to determine SAM50 protein levels. Quantification of protein expression relative to control is indicated above each lane. (B) 5×10^4 transduced and sorted cells per group were plated on MS5 stromal cells and kept in the co-culture for 5 weeks; cultures were demi-depopulated weekly for analysis. Weekly cumulative cell growth is shown for a representative experiment of 4 independent experiments. (C) Suspension cells from MS5 co-cultures as described in panel B were analyzed for the expression of myeloid surface markers at week 2 of co-cullture. Distribution of differentiation (D) Suspension cells from MS5 co-cultures as described in panel B were analyzed for progenitor frequency by CFC assay. 10^4 cells from each co-culture were plated in a CFC assay in methylcellulose in duplicate, and colonies were evaluated 2 weeks after plating. CFC cells were then harvested and 10^5 cells were re-plated to form secondary CFCs. CFU-GM and BFU-E numbers are shown from a representative of 3 independent experiments; error bars indicate standard deviation. (E) LTC-IC frequencies were determined in limiting dilution on MS5 stromal cells. After 5 weeks of culture methylcellulose was added and colonies were scored two weeks later. Poisson statistics were used to calculate LTC-IC frequencies. (F) Suspension cells from the co-cultures as described in panel B were harvested after 14 days and stained with DilC to measure changes in mitochondrial membrane potential. Representative FACS plots and quantification of FACS measurements are shown, represented as changes in MFI relative to control. Average of 3 independent experiments is shown with standard deviation. (G) Suspension cells from the co-cultures as described in panel B were harvested after 14 days and stained with CellROX to measure oxidative stress. Representative FACS plot and quantification of FACS measurements are shown, represented as changes in MFI relative to control. Average of 3 independent experiments is shown with standard deviation. CFU -GM, colony-forming unit-granulocyte-macrophage; BFU-E, burst forming unit-erythroid. * $P<0.05$, *** $P<0.001$.

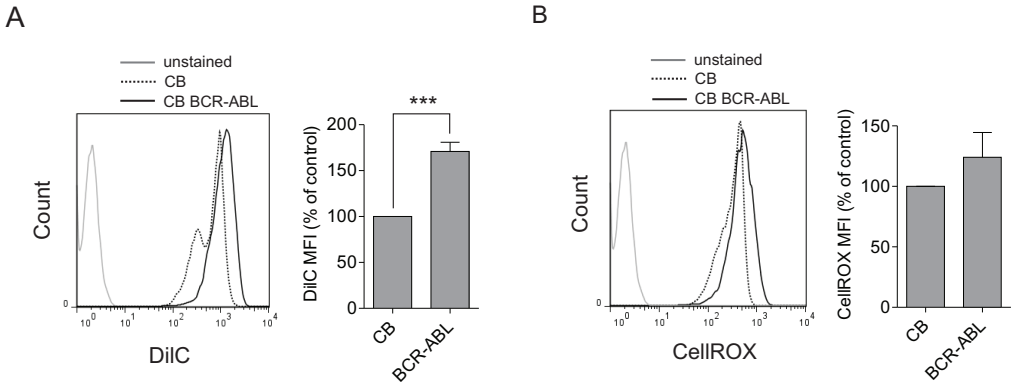


Figure 4. Increased mitochondrial activity of BCR-ABL-transduced human HSPCs. (A) 5×10^4 CB $CD34^+$ stem/progenitor cells transduced either control scrambled shRNA vector (shSCR) or with SAM50-targeting shRNA vectors (shSAM50) as well as 5×10^3 BCR-ABL and shRNA double-transduced cells were sorted and plated on MS5 stromal cells. Suspension cells were harvested after 14 days of co-culture and stained with DilC to measure changes in mitochondrial membrane potential. Representative FACS plot and quantification of FACS measurements are shown, represented as changes in MFI relative to control. Average of 3 independent experiments is shown with standard deviation. (B) Suspension cells from the co-cultures as described in panel A were harvested after 14 days and stained with CellROX to measure oxidative stress. Representative FACS plot and quantification of FACS measurements are shown, represented as changes in MFI relative to control. Average of 3 independent experiments is shown with standard deviation. *** $P<0.001$.

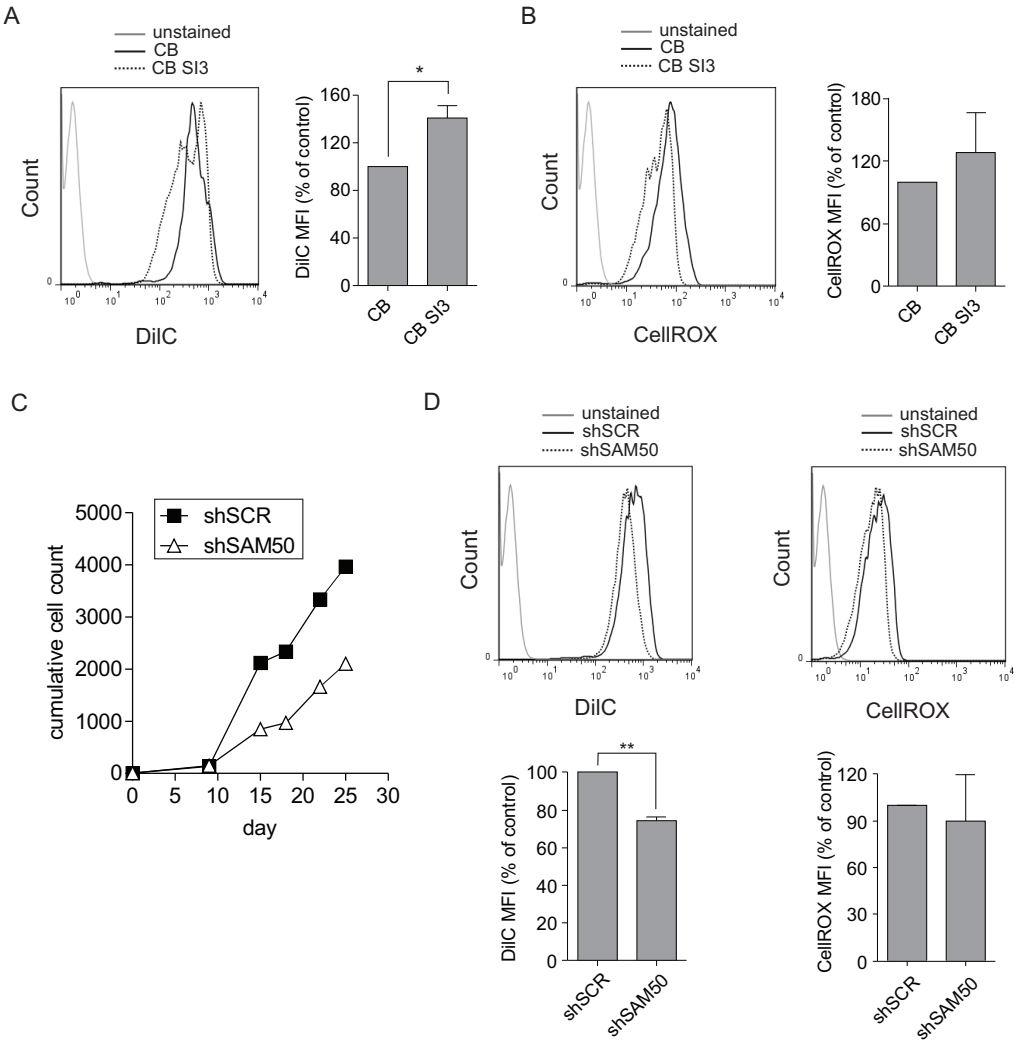


Figure 5. Increased mitochondrial activity and increased sensitivity to SAM50 downregulation in normal HSPCs upon cytokine stimulation. (A) 5×10^4 CB $CD34^+$ stem/progenitor cells were plated on MS5 stromal cells in Gartner's medium with or without the addition of cytokines (20 ng/mL SCF and IL-3). Suspension cells were harvested after 14 days of co-culture and stained with DilC to measure changes in mitochondrial membrane potential. Representative FACS plot and quantification of FACS measurements are shown, represented as changes in MFI relative to control. Average of 3 independent experiments is shown with standard deviation. (B) Suspension cells from the co-cultures as described in panel A were harvested after 14 days and stained with CellROX to measure oxidative stress. Representative FACS plot and quantification of FACS measurements are shown, represented as changes in MFI relative to control. Average of 3 independent experiments is shown with standard deviation. *** $P<0.0001$. (C) CB $CD34^+$ cells were transduced with control shSCR or with shSAM50 vectors, and 5×10^4 transduced and sorted cells per group were plated on

membrane potential and (although not statistically significant) increased ROS levels. This was in accordance with previous findings, where increased mitochondrial biogenesis and increased respiratory rates were shown to be characteristic for leukemic cells [22,38]. Thus, we concluded that an increase in mitochondrial activity of leukemic cells makes them more vulnerable to strategies disrupting mitochondrial functions

Although in the steady state the proliferation and progenitor frequency of normal CB cells in co-culture was not affected by SAM50 downregulation, the replating capacity of progenitor cells was strongly reduced. This suggests that upon stress, possibly due to increased energy requirements, cells rely more strongly upon intact mitochondrial functions. Malignant transformation, with its increased proliferation and metabolic rate, also creates stress within the cells. Moreover, accumulation of ROS results in so-called cancer-associated oxidative stress [15]. Although elevated ROS levels can be advantageous for malignant cells by inducing pro-survival signaling pathways, too high ROS cause detrimental DNA and protein damage [41]. Therefore, disruption of the redox balance in either direction can negatively affect growth and survival of transformed cells.

Addition of cytokines to the co-culture medium not only increased the proliferation rate of normal CB cells, but also rendered them more sensitive to SAM50 downregulation. This effect could simply be a result of a higher proliferation rate that requires more energy produced by the mitochondria, but also a direct effect of cytokine signaling. Several growth factors and cytokines, including IL-3, were found to stimulate ROS production [42,43]. In accordance with that, in our study we found not only increased mitochondrial membrane potential, but also increased, albeit not statistically significant, ROS levels in cytokine-stimulated CB cells. Although the effect of SAM50 downregulation on the growth of CB cells in cytokine-rich cultures was clear, it was less dramatic than in BCR-ABL-transduced CB cells. This suggests that driving cell proliferation is not the only aspect of the oncogenic kinase activity that is responsible for the increased sensitivity to SAM50 downregulation. Elucidating these additional mechanisms that make leukemic cells highly dependent on mitochondrial functions could contribute to more efficient and selective targeting of leukemia.

MS5 stromal cells and kept in the co-culture for 5 weeks, in medium supplemented with cytokines. Co-cultures were demi-depopulated weekly for analysis. Weekly cumulative cell growth is shown for a representative experiment of 3 independent experiments. (D) Suspension cells from co-cultures like described in panel C were harvested after 14 days of co-culture and stained with DilC to measure changes in mitochondrial membrane potential. Representative FACS plot and quantification of FACS measurements are shown, represented as changes in MFI relative to control. Average of 3 independent experiments is shown with standard deviation. (D) Suspension cells from the co-cultures as described in panel C were harvested after 14 days and stained with CellROX to measure oxidative stress. Representative FACS plot and quantification of FACS measurements are shown, represented as changes in MFI relative to control. Average of 3 independent experiments is shown with standard deviation. * $P<0.05$, ** $P<0.01$

ACKNOWLEDGMENTS

The authors would like to thank Dr. Vera Kozjak-Pavlovic from the University of Würzburg, Germany for kindly providing anti-SAM50 antibody, as well as all the members of the Experimental Hematology lab for helpful discussions. We thank Jeanet Dales for help with cord blood isolations. This work was supported by a grant from The Netherlands Organization for Scientific Research (NWO-VIDI 91796312) to JJS.

DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

The authors indicate no potential conflicts of interest.

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