A role for GPx3 in activity of normal and leukemia stem cells

Olivier Herault,1,2,3 Kristin J. Hope,1 Eric Deneault,1 Nadine Mayotte,1 Jalila Chagraoui,1 Brian T. Wilhelm,1 Sonia Cellot,1 Martin Sauvageau,1 Miguel A. Andrade-Navarro,4,5 Josée Hébert,6 and Guy Sauvageau1

1Molecular Genetics of Stem Cells Laboratory, Institute for Research in Immunology and Cancer, University of Montreal, Montreal, Quebec H3C 3J7, Canada
2LNOx team, Centre National de la Recherche Scientifique UMR 7292, Université François Rabelais, 37200 Tours, France
3Service d’Hémato logie Biologique, Centre Hospitalier Régional et Universitaire de Tours, 37000 Tours, France
4Ottawa Hospital Research Institute, Ottawa, Ontario K1H 8L6, Canada
5Computational Biology and Data Mining Group, Max Delbrück Centre for Molecular Medicine, 13125 Berlin, Germany
6Leukemia Cell Bank of Quebec and Division of Hematology, Maisonneuve-Rosemont Hospital, Montreal, Quebec H1T 2M4, Canada

The determinants of normal and leukemic stem cell self-renewal remain poorly characterized. We report that expression of the reactive oxygen species (ROS) scavenger glutathione peroxidase 3 (GPx3) positively correlates with the frequency of leukemia stem cells (LSCs) in Hoxa9+Meis1-induced leukemias. Compared with a leukemia with a low frequency of LSCs, a leukemia with a high frequency of LSCs showed hypomethylation of the Gpx3 promoter region, and expressed high levels of Gpx3 and low levels of ROS. LSCs and normal hematopoietic stem cells (HSCs) engineered to express Gpx3 short hairpin RNA (shRNA) were much less competitive in vivo than control cells. However, progenitor cell proliferation and differentiation was not affected by Gpx3 shRNA. Consistent with this, HSCs overexpressing Gpx3 were significantly more competitive than control cells in long-term repopulation experiments, and overexpression of the self-renewal genes Pdm16 or Hoxb4 boosted Gpx3 expression. In human primary acute myeloid leukemia samples, Gpx3 expression level directly correlated with adverse prognostic outcome, revealing a potential novel target for the eradication of LSCs.

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levels of an additional 170 potential regulators of stem cell activity analyzed (Supplemental Text). In accordance with the Gpx3 overexpression in FLA2, 14/14 tested CpG sequences in the Gpx3 promoter region were methylated in FLB1 and hypomethylated in FLA2 cells (Fig. 1 B). Higher expression of Gpx3 in FLA2 compared with FLB1 cells resulted in increased protein levels (Fig. 1 C) and elevated glutathione peroxidase activity (1,773 ± 127 nmol/min/ml and 1,077 ± 60 nmol/min/ml, respectively; Fig. 1 D).

Flow cytometric comparison of the ROS indicator dichlorodihydrofluorescein diacetate (H2DCFDA) fluorescence revealed a relative reduction in ROS levels in FLA2 compared with FLB1 leukemia (Fig. 2 A), and confocal analysis confirmed these results (Fig. 2 B). Elevated ROS levels also result in lipid peroxidation and formation of malondialdehyde (MDA) protein adducts, and Western blot analyses demonstrated increase in MDA levels in FLB1 compared with FLA2 cells (Fig. 2 C).

Members of the FoxO subfamily regulate intracellular ROS through transcriptional up-regulation of antioxidative enzymes…
in these experiments ranged from 42 to 66%, and because FLA2 cells poorly survive cell sorting, untransduced leukemia-initiating cells were co-transplanted together with transduced cells. This resulted in competitive repopulation experiments in which both the GFP+ shRNA (either Luciferase or Gpx3)-transduced and GFP- untransduced FLA2 cells contributed to leukemia development in vivo. To assess the effect of shRNA treatment on relative contribution of each cell population to clinical leukemia 19 d later, we compared changes in proportions (Fig. 3 B, left) and mean fluorescence intensity (MFI; Fig. 3 B, middle and right) of GFP+ FLA2 cells at two different time points (t = 0 and 19 d). The decrease in proportion of GFP+ FLA2 cells contributing to fulminant leukemia (~19 d) below the values determined for the initial transplant was at least twofold higher after Gpx3 knockdown compared with shLuc control (Fig. 3 B, left). Moreover, the shGpx3-infected FLA2 cells remaining at day 19 were statistically different from those infected with shLuc (Fig. 3 B, middle and right). This result suggests that the only differentially expressed antioxidant enzyme GPx3 may be responsible for the decreased ROS observed in FLA2 leukemia.

**GPx3 promotes LSC in vivo competitiveness**

Correlation between GPx3 levels and LSC frequency could reflect functional dependence of LSC on this enzyme. We exploited retroviruses encoding short hairpin RNAs (shRNAs) and IRES-GFP reporter to explore the in vivo activity of FLA2 cells after GPx3 knockdown (Fig. 3 A). Gene transfer in these experiments ranged from 42 to 66%, and because FLA2 cells poorly survive cell sorting, untransduced leukemia-initiating cells were co-transplanted together with transduced cells. This resulted in competitive repopulation experiments in which both the GFP+ shRNA (either Luciferase or Gpx3)-transduced and GFP- untransduced FLA2 cells contributed to leukemia development in vivo. To assess the effect of shRNA treatment on relative contribution of each cell population to clinical leukemia 19 d later, we compared changes in proportions (Fig. 3 B, left) and mean fluorescence intensity (MFI; Fig. 3 B, middle and right) of GFP+ FLA2 cells at two different time points (t = 0 and 19 d). The decrease in proportion of GFP+ FLA2 cells contributing to fulminant leukemia (~19 d) below the values determined for the initial transplant was at least twofold higher after Gpx3 knockdown compared with shLuc control (Fig. 3 B, left). Moreover, the shGpx3-infected FLA2 cells remaining at day 19 were statistically different from those infected with shLuc (Fig. 3 B, middle and right). This result suggests that the only differentially expressed antioxidant enzyme GPx3 may be responsible for the decreased ROS observed in FLA2 leukemia.

**Figure 2.** The ROS level is decreased in FLA2 cells. (A) Intracellular ROS levels in FLA2 and FLB1 evaluated by flow cytometry using H2DCFDA probe immediately after extracting marrow from leukemic mice. Open histograms, DMSO (vehicle control); filled histograms, H2DCFDA. (B) Confocal microscopy of FLA2 and FLB1 cells stained with H2DCFDA. 20 cells of each leukemia were randomly chosen and classified according to H2DCFDA fluorescence. The corresponding phase-contrast pictures are presented. (C) Western blot of MDA-modified protein adducts in FLA2 and FLB1 cells. H2O2-treated FLA2 cells were used as a positive control and α-tubulin as a loading control. Asterisks indicate major MDA protein adduct species. The dotted line shows margins of two blots shown in a merged image.

(Kops et al., 2002; Tothova et al., 2007). FoxO-deficient HSCs were reported to overexpress several antioxidant enzymes such as Sod1, Sod3, Prdx6, and Gpx4, but not Gpx3 (Tothova et al., 2007). FLA2 and FLB1 leukemias expressed comparable levels of Foxo3a, Foxo4, and Foxo6, whereas Foxo1 was not expressed (Supplemental Text), indicating that FoxO induction is likely not responsible for differential ROS (Supplemental Text) and suggesting that the only differentially expressed antioxidant enzyme GPx3 may be responsible for the decreased ROS observed in FLA2 leukemia.

**Figure 3.** Gpx3 depletion decreased LSC competitiveness. Left: effect of the RNAi-mediated depletion of Gpx3 on the competitiveness of FLA2 leukemic cells. 2×10⁵ fresh FLA2 BM cells were co-cultured with shLuc or shGpx3 retrovirus-producing GP+E-86 cells. After infection (d0), cells were partitioned between flow cytometric analysis of GFP+ frequency and transplantation of unsorted cells into sublethally irradiated recipients (shLuc, n = 12; shGpx3, n = 16). Contribution of GFP+ (shRNA-transduced) cells to leukemia was determined on day 19. Results are shown as normalized values: the GFP+ frequency in inoculum was considered 100% (dotted line, d0), and the day 19 GFP+ frequency was calculated as a percentage of that value. Horizontal bars represent mean values determined for each experimental group. Middle: day 19 MFI of GFP- cells in recipients of shLuc- and shGpx3-infected FLA2 leukemic cells. The dotted line represents MFI of GFP+ cells in shLuc and shGpx3 inocula. Horizontal bars represent mean values determined for each experimental group. Right: GFP fluorescence intensity in representative recipients of shLuc- or shGpx3-infected FLA2 cells. The inset demonstrates comparable MFI GFP fluorescence in shLuc and shGpx3 inocula. *, P = 0.0037 and P = 0.0015, respectively (Wilcoxon’s test).
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...displayed a significant reduction in MFI of GFP compared with controls (Fig. 3 B, middle and right). These results indicate that GFP<sup>high</sup> cells were selectively depleted (Fig. 3 B, right, inset), and suggest that GPx3 is critical for the competitiveness of LSCs.

We quantified GPX3 expression in 48 acute myeloid leukemia patients categorized into cytogenetically and molecularly defined risk subgroups designated as favorable, intermediate, and adverse (Mrózek et al., 2007). Within the AML samples, expression of GPX3 increased with the severity of disease, and the highest expression levels were associated with adverse risk group of patients (P = 0.019; Fig. 4). The worst clinical outcomes were thus observed in AML enriched for phenotypically defined LSC (van Rhenen et al., 2005) and high GPX3 levels, indicating that GPX3 may be relevant for human leukemia.

Gpx3 influences normal HSC activity

Adult BM-derived HSCs are less proliferative than their fetal liver (FL)-derived counterparts (Rebel et al., 1996). Quantitative RT-PCR (qRT-PCR) assays revealed that HSC isolated from BM of 3- and 4-wk-old mice express 39- to 6-fold, respectively, lower GPx3 levels than HSC sorted from embryonic day 14.5 (E14.5) FL (Fig. 5 A), suggesting a correlation between GPx3 expression and HSC activity. Interestingly, GPx3 levels were also elevated in BM HSC/progenitor cells...
engineered to overexpress genes associated with self-renewal such as Hoxb4, NA10HD, Klf10, and Prdm16 (Sauvageau et al., 1995; Ohta et al., 2007; Deneault et al., 2009). In these transduced cells, levels of Gpx3 expression increased 3.2- to 19.2-fold compared with controls (Fig. 5 B).

We next infected BM HSC/progenitor cells with retroviruses carrying two different Gpx3 hairpins with the ability to achieve ~95% Gpx3 knockdown (Fig. 5 C, left). Clonogenic progenitor cell activity was not affected by this decrease in Gpx3 expression (Fig. 5 C, right), and there was no increase in apoptosis in the shGpx3 cell population compared with shLuc controls (not depicted). shRNA-mediated Gpx3 silencing, however, dramatically impeded the ability of transplanted HSC to repopulate the hematopoietic system of recipient mice at early (8 wk) and late (39 wk) time points after transplantation (Fig. 5 D). We also transplanted the shGpx3- and shLuc-transduced 5-FU-derived BM cells and analyzed their ability to home in the BM 48 h after injection. These experiments showed no difference between homing abilities of shGpx3- and shLuc-transduced cells. Together, these results identify Gpx3 as a critical regulator of short- and long-term repopulating (LTR) HSC.

In reciprocal gain-of-function experiments, BM HSC/progenitor cells engineered to overexpress Gpx3 showed a marked competitive advantage over controls when transplanted after a 7-d ex vivo culture step (Fig. 5 E). Proviral integration analyses of six recipients confirmed polyclonal hematopoiesis (Fig. 5 F), thus ruling out the possibility of insertional mutagenesis. Moreover, some mice (#2570 and #2571; Fig. 5 F) were reconstituted by the same clones, indicating that self-renewal occurred in vitro before transplantation. GFP+ Gpx3-transduced cells contributed to production of lymphoid and myeloid peripheral blood leukocytes at >30 wk after transplantation, confirming the multipotential nature of the reconstituting clones (Fig. 5 G). These mice were hematologically normal and showed no evidence of myeloproliferation or leukemia. Together, these results indicate that Gpx3 enhances HSC expansion ex vivo possibly through modulation of self-renewal activity. Unfortunately, such gain-of-function experiments were not possible with our FLB1 leukemia model as we have not yet identified the in vitro conditions that would allow their effective transduction.

In summary, identification of an antioxidant enzyme such as Gpx3 acting as a stem cell activity factor in leukemia raises the possibility that other ROS scavenging systems have regulatory importance in other CSS. A low ROS level in breast CSCs has recently been associated with increased expression of Foxo1 and the glutathione biosynthesis genes Gis and Gelm (Diehn et al., 2009). In normal hematopoiesis, ROSlow HSCs have a higher self-renewal potential than ROShigh HSCs (Jang and Sharkis, 2007), whereas loss of ATM (ataxia telangietasia mutated) or a FoxO transcription factor correlates with elevated ROS levels and limits self-renewal capacity of HSC during serial transplantations (Ito et al., 2004, 2006; Tothova et al., 2007). ROS can function as signaling molecules in several cell types (Finkel and Holbrook, 2000; Owusu-Ansah et al., 2008; Takeda et al., 2008) and can modulate the activation of signal transduction pathways involved in cellular proliferation and differentiation (Sundaresan et al., 1995; Sattler et al., 1999; Owusu-Ansah and Banerjee, 2009).

Although the current study highlights the importance of Gpx3 in normal and leukemia hematopoiesis, the implication of ROS as the key modulator of Gpx3 activity has not been established. This is particularly critical in the context that ROS scavenger (N-acetyl-l-cysteine treatment) and p38 inhibition failed to consistently increase LSC frequency of FLB1 leukemia in vivo (unpublished data). Nonetheless, our results indicate that in some conditions (Hoxb4 or Prdm16 gain of function) Gpx3 expression may represent a converging pathway that determines the activity of normal and leukemic stem cells.

MATERIALS AND METHODS

Mice. Donor C57BL/6:PeP3b (Ly5.1) and recipient C57BL/6 (Ly5.2) mice were bred and maintained as previously described (Kroon et al., 1998). Experimental procedures were reviewed and approved by the University of Montreal animal ethics committee (Comité de Déontologie de l’Expérimentation sur les Animaux de l’Université de Montréal).

Human cells. AML tumor samples and normal marrow samples were obtained from the Quebec Leukemia Cell Bank (Montreal, Quebec) and the Orthopedic Surgery Department of the University Hospital of Tours (Tours, France), respectively. All these cells were obtained according to approved protocols of the institutional review boards after informed consent. Patients were classified in three risk groups for cytogenetic and molecular abnormalities (Mrózek et al., 2007).

Isolation of mouse hematopoietic cell subpopulations. Cells were first purified for Sca1 using magnetic activated cell sorting as per the manufacturer’s instructions (Miltenyi Biotec). Further antibody staining and sorting to purify the c-Kit+, lineage− (CD45R−, CD3−, Gr-1−, Ter119−) population were performed as described previously (Okada et al., 1992). The LTR-HSC from BM (c-Kit+Lin−Sca1+CD150+CD48−, KLS SLAM) or E14.5 d.p.c. FL (CD150+CD48+Sca1+Mac1−Lin−, SLAM Sca1+Mac1+) and HSC/progenitor cells (CD150+CD48 Lin−) from BM were sorted using FACs-Aria cytometer (BD).

Retroviral infection and transplantation. Infection and transplantation procedures were performed as previously described (Thorsteinsdottir et al., 1999). Infection medium consisted of DMEM, 15% FCS, 100 ng/ml IL-11, 50 ng/ml Flt-3 ligand, 100 ng/ml SF, 10 μg/ml ciprofloxacin (SeroLogicals), and 6 μg/ml polybrene (hexadimethrine bromide; Sigma-Aldrich).

Methylcellulose cultures. Progenitor assays were plated and scored as previously described (Kroon et al., 1998).

Competitive repopulation unit enumeration assays. Stem cell quantification assays were performed essentially as previously described (Antonchuk et al., 2001).

Fluorescence studies. The studies were performed with monoclonal antibodies specific for the following: Sca-1 (D7), Mac1 (M1/70), and Gr-1 (RB6-8C5; BD); kit (2B8), CD48 (HM48-1), and CD3 (17A2), eBioscience); and CD11b (M1/70), and TER-119 (BioLegend). Staining with H2DCFDA was performed according to the manufacturer’s instructions (Invitrogen) using fresh, not purified leukemic cells immediately after BM harvest. The peak excitation wavelength for oxidized H2DCFDA was 488 nm, and emission was 525 nm. Flow cytometry and confocal analyses were performed using the Aria cytometer (BD).
using an LSR II cytometer (BD) and an LSM 510 laser-scanning confocal microscope (Carl Zeiss), respectively.

**DNA and RNA preparation.** Genomic DNA was isolated using DNAzol and total cellular RNA with TRIzol, according to the manufacturer’s instructions (Invitrogen).

**Southern blot analysis.** All blots were prepared and analyzed as previously described (Kroon et al., 1998).

**Bisulfite conversion and sequencing.** Genomic DNA was converted using the methylSEQuencer Bisulfite Conversion Kit (Applied Biosystems) according to the manufacturer's instructions. The Gpx3 region was amplified by PCR and sequenced using an M13 tail (BigDye Terminator v3.1; Applied Biosystems). Purified sequencing products were analyzed on a 3730 Genetic Analyzer (Applied Biosystems).

**Hybridization.** RNA samples were sent to the microarray facility at the Ottawa Hospital Research Institute (Ottawa, ON), where the RNA was reverse transcribed, labeled, and hybridized to Affymetrix 430A arrays according to manufacturer’s protocols.

**Affymetrix gene expression profiling.** Affymetrix CEL files from biological triplicates for FLA2 and FLB1 were normalized using either RMA or GC-ERA, using the LIMMA (Linear Models for MicroArray data) package for R/Bioconductor. Normalized data were then used to compare the linear model to derive differentially expressed genes, or exported to perform SAM (significance analysis of microarrays) analysis. SAM analysis on both normalized datasets was performed using version 3.01 of the SAM software, with 100 permutations and a two-sided minimum change in both cases. Using delta calibration values of 1.236 (GC-ERA) and 0.567 (RMA), lists of 33 or 31 genes which showed differential expression (22 genes in common) were generated with false discovery rates of 2.71 and 3.31%, respectively. The ArrayExpress accession no. is E-MEXP-1597.

**qRT-PCR.** qRT-PCR was performed using two strategies: the SYBR green dye and Universal ProbeLibrary assays designed with the ProbeFinder software (Roche) as described using Hprt, Asb3, GAPDH, or HPRT as endogenous controls. Ct values were determined using the advanced relative quantification algorithm for each target (Ct(target)) and endogenous control reference genes (Ct(reference)). The expression level of the target gene was expressed as ΔCt value (ΔCt = Ct(target) – Ct(reference)) and calculated as RQ (ΔCt(target population)/ΔCt(endogenous population)). Calibrators used for calculation of various RQ values are indicated in the corresponding figure legends. To ensure that our qRT-PCR analyses accurately reflected expression of genes encoding antioxidant enzymes in the LTR-HSC compartment, only sorted HSC populations comprised of ~50% of LTR-HSC as determined by competitive CRU were processed for cDNA synthesis and gene expression studies presented in Fig. 5 A.

**Protein extraction and immunoblot.** Cells were lysed in 10 mmol/liter Heps, pH 7.9, 10 mmol/liter KCl, 0.1 mmol/liter EDTA, 0.1 mmol/liter EGTA, and 10% (vol/vol) NP-40 containing protease inhibitors. Western blot analyses were done as previously described (Krosl and Sauvageau, 2000) using antibodies against Gpx3 (R&D Systems) and MDA (Abcam).

**Glutathione peroxidase assay.** The enzymatic activity of glutathione peroxidase in the spleen of mice was quantified using the Glutathione Peroxidase Assay kit (Cayman Chemical Company) according to the manufacturer’s recommendations.

**Construction of shRNA retroviral vectors.** Two shRNAs were selected as single-stranded oligonucleotides also incorporating mtr-l0 flanking arms using the RNAs Central shRNA psi2 design tool (G. Hannon, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY). Amplitcons were digested with Xhol–EcoRI (Invitrogen) and subcloned into the LMP vector according to the manufacturer’s guidelines (Invitrogen). Confirmatory sequencing was performed on all cloned shRNAs: shGpx3 #5, 5'-TGCTGGTGACCGTGCGCTTTCATCTTCCGTGAAAGACACCA-GATGTTGCTTCTCCAGAAAAGTGTAGTCCTACTGCCTG-AGA-3'; shGpx3 #6, 5'-TGCTGGTGACCGTGCGCTTTCATCTTCCGTGAAAGACACCA-GATGTTGCTTCTCCAGAAAAGTGTAGTCCTACTGCCTG-AGA-3'; and shLuc, 5'-TGCTGGTGACCGTGCGCTTTCATCTTCCGTGAAAGACACCA-GATGTTGCTTCTCCAGAAAAGTGTAGTCCTACTGCCTG-AGA-3'.

**Apoptosis assay.** Phosphatidylserine externalization was analyzed 4 d after retroviral infection by labeling GFP+ cells with annexin V–Alexa Fluor 350 and propidium iodide. Flow cytometry analyses were performed using an LSR II cytometer.

**Homing assay.** After retroviral infection, Ly5.1+ cells were transplanted via tail vein injection into irradiated (1,600 cGy, 137 Cs gamma radiation) C57BL/6j (Ly5.2) mice. A small aliquot of cells was plated at this time in methylcellulose to assess the percentage of transduced CFUs in the inoculum (see Methylcellulose cultures). 4 d after transplant, recipient BM was harvested and the percentage of GFP+ Ly5.1+ cells was assessed by flow cytometry with the acquisition of a minimum of 10⁶ events (LSR II cytometer). BM was also plated in methylcellulose for later enumeration and scoring of GFP+ and GFP− CFUs (see Methylcellulose cultures). Statistical analyses. Statistical analyses were performed using Wilcoxon’s test. The level of significance was set at 0.05.

**Online supplemental material.** Fig. S1 shows representative images of spleens from recipients of FLA2 and FLB1 cells at 19 d after transplantation. Table S1 shows FLA2 gene expression profiling. Online supplemental material is available at www.jem.org/cgi/content/full/jem.20102386/DC1.

**REFERENCES**

