

Title: Enhanced Nox1 expression and oxidative stress resistance in c-kit-positive hematopoietic stem/progenitor cells

(Running head: Redox regulation in stem cells)

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Abstract

Although stem cells are generally thought to be resistant to oxidative stress, the fact and in detail molecular mechanism are still to be clearly identified. We herein tried to understand the overall characterization of redox regulatory signaling in hematopoietic stem cells. We purified c-kit-positive hematopoietic stem/progenitor cells from the bone marrow of healthy mice, and then evaluated their redox regulatory property. Compared to the c-kit-negative matured mononuclear cells, c-kit-positive stem/progenitor cells showed lower basic levels of intracellular reactive oxygen species, faster clearance of the accumulated intracellular reactive oxygen species, and higher resistant to oxidative stress. An overall view on the gene expression profile associated with redox regulation showed to be widely differed between cell types. We confirmed that the c-kit-positive stem/progenitor cells expressed significantly higher of Nox1 and catalase, but less of lactoperoxidase than these matured mononuclear cells. Our data suggests that stem cells keep specific redox regulatory property for defending against oxidative stress.

Introduction

Adult stem cells have widely been identified in different tissues/organs, and known to persist throughout the lifetime for maintaining the homeostasis of organism, including the physiological turnover and the repair/regeneration in case of damage [1]. Several instructive genetic mouse models have recently demonstrated the unique susceptibility of stem cells to perturbations in metabolic or redox homeostasis [2-4]. In contrast, many recent studies have found that these adult stem cells likely to be highly resistant to various stresses, including oxidative stress [5, 6]. Considering the critical roles in maintaining the homeostasis of organism for long-term lifetime, stem cells are generally thought to be specialized in their redox regulatory signaling for defending against endogenous and exogenous damages/stresses. However, the sensitivity and in detail molecular mechanisms on oxidative resistance of stem cells are still to be clearly identified in comparison with these matured tissue cells.

Reactive oxygen species (ROS) are generally known to play as either physiological or pathological roles in different types of cells [7]. Appropriate ROS levels are required for physiological cellular functions and maintaining genomic stability, but excess accumulation of ROS may induce oxidative stress to damage the cells [4, 8, 9]. It has been reported that hematopoietic stem/progenitor cells have relatively low level of endogenous ROS when compared with their mature progeny [10, 11], which seems to play critical roles in maintaining the quiescence and “stemness” [12, 13], because the increase of ROS level could induce the proliferation, differentiation, and maturation of stem cells [14, 15]. The relatively low ROS level in stem cells likely to associate with the up-regulated expression of numerous enzymes and multiple glutathione-independent antioxidants [16]. Functional loss of redox regulatory relevant genes, such as FOXO3a and Mdm2 has been found to contribute an increased intracellular ROS levels and the self-renewal defection of hematopoietic stem cells in animal models [17, 18]. However, the property on redox regulatory signaling in stem cells has not yet been well understood.

By a directly comparison on the redox regulatory property in the purified c-kit⁺ hematopoietic stem/progenitor cells with that of the c-kit⁻ matured mononuclear cells from bone marrow of healthy mice, we tried to uncover the overall characterization on redox regulatory signaling in stem cells.

Materials and methods

Animals

We used 12-week-old male C57BL/6 mice (SLC, Japan) for the present study. All experiments were approved by the Institutional Animal Care and Use Committee of Nagasaki University, and experiments were performed in accordance with the institutional and national guidelines.

Separation of c-kit⁺ stem/progenitor cells and c-kit⁻ matured mononuclear cells from bone marrow

Bone marrow cells were collected from the femur and tibia of healthy mice. The bone marrow mononuclear cells (BM-MNCs) were isolated by density gradient centrifugation [19]. The freshly collected BM-MNCs were then incubated with anti-mouse CD117 (c-kit) antibody (Miltenyi Biotec) for 30 min [19]. After washing, the c-kit⁺ stem/progenitor cells and c-kit⁻ matured mononuclear cells were separated by using the Magnetic Cell Sorting system (autoMACS, Miltenyi Biotec, Auburn, CA). The purity of the c-kit⁺ cells was around 90%, and the viability was more than 99%.

Detection of intracellular ROS levels

To measure the intracellular ROS levels, the freshly purified c-kit⁺ cells (n=5) and c-kit⁻ cells (n=5) from different mice were seeded on 96-well culture plate at a density of 1×10^4 cells/well in 100 μ l IMDM 1640 medium supplemented with 10% fetal bovine serum (HyClone) and incubated at 37°C in 5% CO₂ for 12 hours. Cells were then incubated with 10 μ M CM-H₂DCFDA (Molecular Probes Inc.), at 37°C for 30 minutes [20]. After washing, the fluorescence intensity in each well was measured by plate reader (VICTOR™ X3 Multilabel Plate Reader, PerkinElmer Inc., Waltham, Massachusetts, USA). We also stimulated the c-kit⁺ cells and c-kit⁻ cells with 100 μ M H₂O₂ for 1 hour, and then investigated the accumulation and clearance of ROS in cells after stimulation.

Evaluation on oxidative stress resistance

The freshly purified c-kit⁺ cells (n=5) and c-kit⁻ cells (n=5) were stimulated with 50 and 200

μM H_2O_2 for 2 hours. Cells were then labeled with propidium iodide (PI) to detect the dead cells. After twice washing, apoptotic cells were also staining with ANNEXIN V-FITC Kit according to the manufacturer's instructions (BECKMAN COULTER). Quantitative flow cytometry analysis was performed using a FACSCalibur (Becton Dickinson). We analyzed the acquired data using Cell Quest software (Becton Dickinson) [19, 20].

Mouse Oxidative Stress and Antioxidant Defense Pathway Finder RT2 Profiler PCR array

Total RNA was isolated from freshly purified c-kit⁺ cells (n=5) and c-kit⁻ cells (n=5), by using RNeasy Mini Kit (Qiagen). After the generation of cDNA using RT2 First Strand Kit (SABiosciences), PCR array was done according to the manufacturer's instructions (SABiosciences), and a total of 84 genes involved in antioxidants and ROS metabolism was included in array (*Supporting information Table 1*). Data from 5 separated samples of c-kit⁺ cells and c-kit⁻ cells was calculated for the mean fold change of expression and significance by using web-based data analysis program (SABiosciences) (*Supporting information Table 1*).

Western blotting

The expression levels of SOD1, SOD2, and catalase were measured by Western blotting using Mini-PROTEAN[®] Tetra Cell and Trans-Blot[®] Turbo[™] Transfer System (BIO-RAD) according to the instruction manual. Briefly, the total proteins purified from the c-kit⁺ and c-kit⁻ cells were separated by SDS-PAGE gels, and then transferred to PVDF membranes. After blocking, the membranes were incubated with primary antibodies against SOD1 (500-fold dilution, Santa Cruz Biotechnology, Inc.), SOD2 (500-fold dilution, Santa Cruz Biotechnology, Inc.), catalase (500-fold dilution, Santa Cruz Biotechnology, Inc.), or β -actin (5000-fold dilution, Sigma-Aldrich), followed by the appropriate horseradish peroxidase-conjugated secondary antibodies. The expression was visualized using an enhanced chemiluminescence detection kit, and semi-quantitative analysis was done by measuring the density of bands using ImageQuant LAS 4000mini (GE Healthcare Life Sciences).

Statistical analyses

All results are presented as the means \pm SD. The statistical significance among groups was determined by one-way analysis of variance (ANOVA) followed by post hoc test, but statistical significance between two groups was done by unpaired *t*-test (Dr. SPSS II, Chicago, IL). Differences were considered significant when $p < 0.05$.

Results

Different intracellular ROS level and oxidative stress resistance between c-kit⁺ and c-kit⁻ cells

The basic intracellular ROS levels in c-kit⁺ and c-kit⁻ cells were measured from the freshly isolated cells after incubation at 37°C in 5% CO₂ for 12 hours. In compare with the c-kit⁻ cells, these c-kit⁺ stem/progenitor cells had relatively lower ROS level ($p < 0.01$, **Fig. 1A**). After 1 hour stimulation with 100 μ M H₂O₂, the intracellular ROS levels were significantly increased to a comparable levels in both c-kit⁺ and c-kit⁻ cells ($p < 0.01$, **Fig. 1B**). Although the intracellular ROS was returned to the level that close to baseline in the c-kit⁺ stem/progenitor cells within 18 hours after stimulation ($p = 0.28$ vs. baseline, **Fig. 1B**), significant accumulation of ROS was still observed in the c-kit⁻ mononuclear cells at 18 hours after stimulation ($p < 0.05$ vs. baseline, **Fig. 1B**).

We also investigated the survival of cells after oxidative stress stimulation. We found that the stimulation with either 50 or 200 μ M H₂O₂ significantly induced more cell death and apoptosis of c-kit⁻ cells than that of c-kit⁺ cells (**Fig. 2**), suggested a higher oxidative stress resistance of c-kit⁺ stem/progenitor cells in compared with their mature progeny of the c-kit⁻ mononuclear cells.

Expression of genes involved in antioxidants and ROS metabolism

By pathway focused PCR array analysis, we widely compared the expression of genes involved in antioxidants and ROS metabolism between the c-kit⁺ and c-kit⁻ cells (**Supporting information Table 1**). The top 20 genes that up- or down-regulated were presented in Figure 3 (**Fig. 3A**). We noticed that some genes involved in superoxide metabolism, including *Nox1* and *Nox1*, were up-regulated in the c-kit⁺ stem/progenitor cells. However, the expression of genes associated

other peroxidases, including the *LPO*, was down-regulated in the c-kit⁺ stem/progenitor cells. We have also confirmed these data by semi-quantitative RT-PCR analysis (*Fig. 3B*).

Expression of antioxidant enzymes of SOD1, SOD2, and catalase

Western blot analysis showed that the expression of antioxidant enzymes of SOD1 and SOD2 did not significantly differ between the c-kit⁺ stem/progenitor cells and the c-kit⁻ matured mononuclear cells (*Fig. 4A, B*). However, the expression of catalase was significantly higher in the c-kit⁺ cells than the c-kit⁻ cells ($p < 0.01$, *Fig. 4C*).

Discussion

Although ROS can serve as critical signaling mediators and antimicrobial host defenses [7, 21], the excessive accumulation of ROS is generally known to induce the cellular damage that eventually lead to aging, cardiovascular diseases, inflammation, and carcinogenesis [22]. To protect against oxidative stress-induced injuries, all mammalian have been developed powerful antioxidant mechanisms for maintaining redox homeostasis. Therefore, the understanding on the complex network of redox regulation will be helpful for the prevention and treatment of various diseases and disorders.

As the small population tissue-specific stem/progenitor cells has been well recognized to play critical roles in carcinogenesis and the repair/regeneration of damaged tissues [1, 21, 23, 24], redox regulation signaling in stem cells warrants specific attention in the past decade. A number of recent studies have reported the important role of redox homeostasis in regulating the self-renewal, differentiation, and genomic stability of stem cells [2-6, 8-18]. It has demonstrated that moderately high H₂O₂ levels promote the differentiation of hematopoietic progenitors through JNK and FOXO activation [13, 14]. We have also recently found that ROS likely plays dual roles in the genomic stability of stem cells [8], of that, a physiological level of ROS is required for effective DNA repair, but high ROS induces DNA damage. However, the characterization of redox regulatory property in stem cells has not yet been well understood.

In this study, we tried to investigate the redox regulatory property in the c-kit⁺ stem/progenitor

cells by directly comparison with the c-kit⁻ matured mononuclear cells from the bone marrow of healthy mice. Agreed well with previous study [10], the basic ROS level in c-kit⁺ stem/progenitor cells was detected relatively lower than the c-kit⁻ matured mononuclear cells. Although the stimulation with H₂O₂ increased the intracellular ROS levels to a comparable levels in both of the c-kit⁺ and c-kit⁻ cells, these c-kit⁺ stem/progenitor cells showed a faster clearance of accumulated ROS. This provides a reasonable explanation to the higher resistant to oxidative stress. Therefore, it would be critical to understand how the stem cells were metabolically differed from the more differentiated cells.

By screening the expression of genes involved in antioxidants and ROS metabolism, we found that the gene-expression profiling was widely varied between the c-kit⁺ and c-kit⁻ cells. Our data showed that the c-kit⁺ stem/progenitor cells were highly expressed with Nox1 and Noxa1 than the c-kit⁻ mononuclear cells. Redox homeostasis is known to be regulated by a number of oxidases, among which the plasma membrane NADPH oxidase (NOX) is recognized to be one of the major players [15]. Previous study has demonstrated that bone marrow derived hematopoietic stem cells express the catalytic subunits of Nox1, Nox2, and Nox4 isoforms of the NADPH oxidase family along with the complete battery of the regulatory subunits of Noxo1 and Noxa1, which contribute to produce constitutively low levels of ROS [25]. It has been suggested that the existence of different types of ROS-generators might provide a fine mechanism of tuning redox signaling to control the delicate balance between cell growth, proliferation, and differentiation of hematopoietic stem cells [26]. This provides reasonable explanation on the higher expression of Nox1 and Noxa1 in these c-kit⁺ hematopoietic stem/progenitor cells than the matured c-kit⁻ mononuclear cells.

Interestingly, some genes involved in antioxidants and ROS metabolism, such as lactoperoxidase (Lpo), was largely down-regulated in the c-kit⁺ stem/progenitor cells. The Lpo is functionally known to play a key role in the innate immune defense by oxidizing thiocyanate into hypothiocyanite [27], which serve to kill the bacterium and fungi. Therefore, the relatively lower Lpo in the c-kit⁺ stem/progenitor cells from bone marrow more likely due to the specificity of enhanced Lpo expression in the matured c-kit⁻ mononuclear cells for immune defense, rather than the down-regulation of Lpo in general in stem cells.

Peroxiredoxin 1 (Prxd1), an antioxidant that well known to protect cells from metabolically produced ROS, was also down-regulated in the c-kit⁺ stem/progenitor cells. It has recently been reported that the incubation of Prxd1 with immature bone marrow-derived dendritic cells resulted in TLR4-dependent secretion of TNF-alpha and IL-6 and dendritic cell maturation, suggest that Prxd1 may act as danger signal similar to other TLR4-binding chaperone molecules such as HSP72 [28]. Similarly, the relatively lower expression of Prxd1 in c-kit⁺ stem/progenitor cells may also simply due to the highly expression of Prxd1 in the matured c-kit⁻ mononuclear cells, rather than in general from the view of stem cell biology.

In conclusion, c-kit⁺ hematopoietic stem/progenitor cells likely resistant to oxidative stress when compared with the more differentiated c-kit⁻ mononuclear cells in bone marrow. Although widely different in the expression of genes involved in antioxidants and ROS metabolism was detected between two cell types, it is still required to understand the significance of redox regulatory property in stem cell biology.

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Figure Legends

Fig. 1. *ROS levels in c-kit⁺ stem/progenitor cells and c-kit⁻ matured mononuclear cells.* **A)** Cells were incubated for 12 hours and then labeled with 10 μ M CM-H₂DCFDA for 30 minutes. The ROS level was evaluated by measuring the fluorescence intensity within cells. **B)** Cells were stimulated with 100 μ M H₂O₂ for 1 hour and then followed the changes of ROS levels at 0, 1, 2, 3, and 18 hours after stimulation.

Fig. 2. *Oxidative stress resistance of c-kit⁺ stem/progenitor cells and c-kit⁻ matured mononuclear cells.* Cells were incubated with 50 and 200 μ M H₂O₂ for 2 hours and then stained with PI (**A**) and Annexin V (**B**) for evaluating cell death and apoptosis by flow cytometry.

Fig. 3. *The expression of genes that involved in antioxidants and ROS metabolism in c-kit⁺ stem/progenitor cells and c-kit⁻ matured mononuclear cells.* **A)** The top 20 genes that up- or down-regulated in c-kit⁺ stem/progenitor cells. **B)** Quantitatively PCR analysis on the top two genes of Nox and Lpo.

Fig. 4. *Western blot analysis on SOD1, SOD2, and the catalase.* Although the expression of catalase was significantly higher in c-kit⁺ stem/progenitor cells than the c-kit⁻ matured mononuclear cells (**A**), the expression of SOD1 (**B**) and SOD2 (**C**) was comparable between two cell types.

Fig 1.

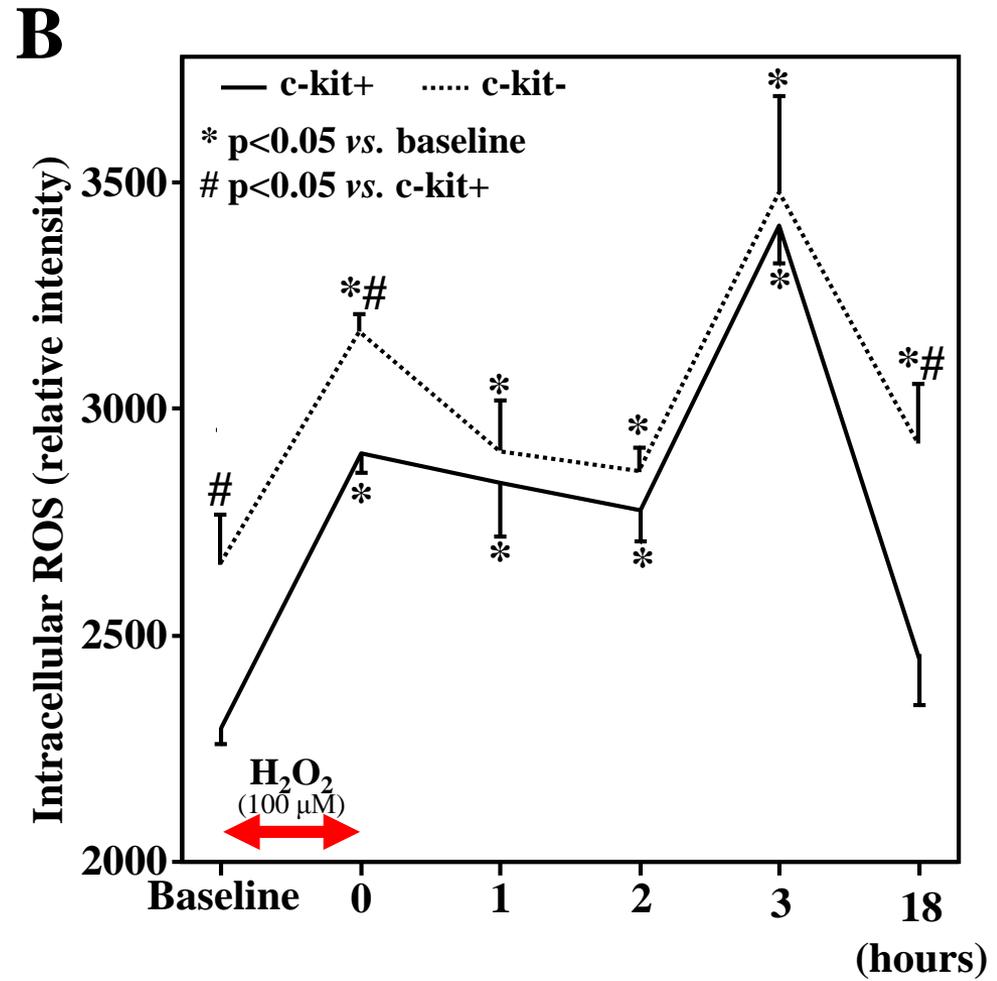
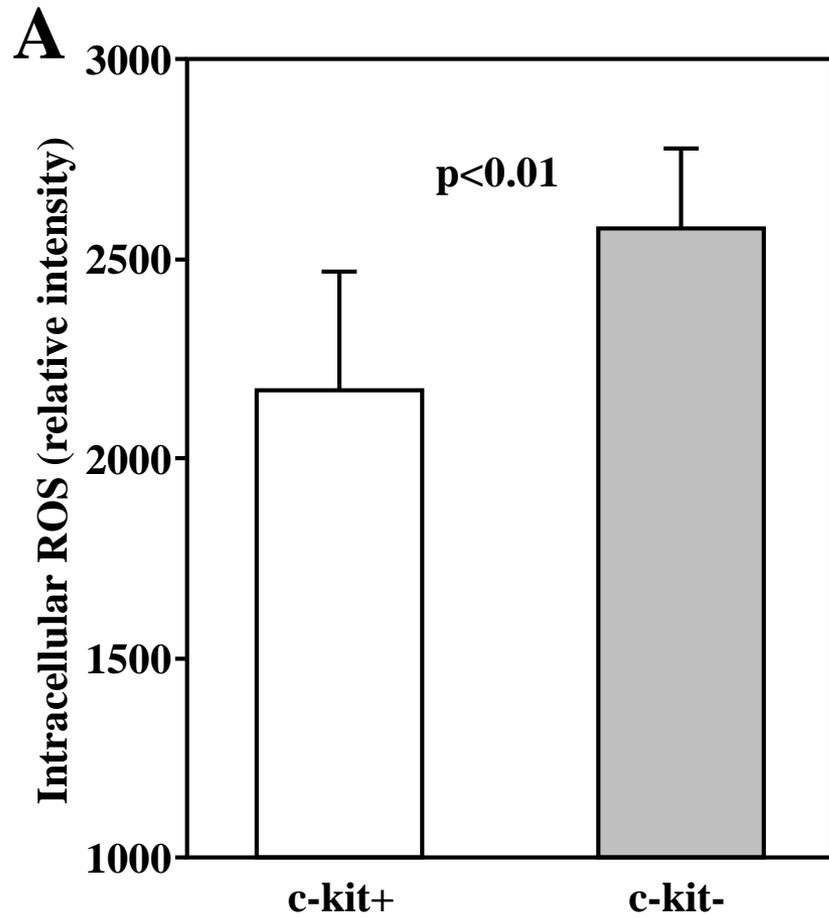


Fig 2.

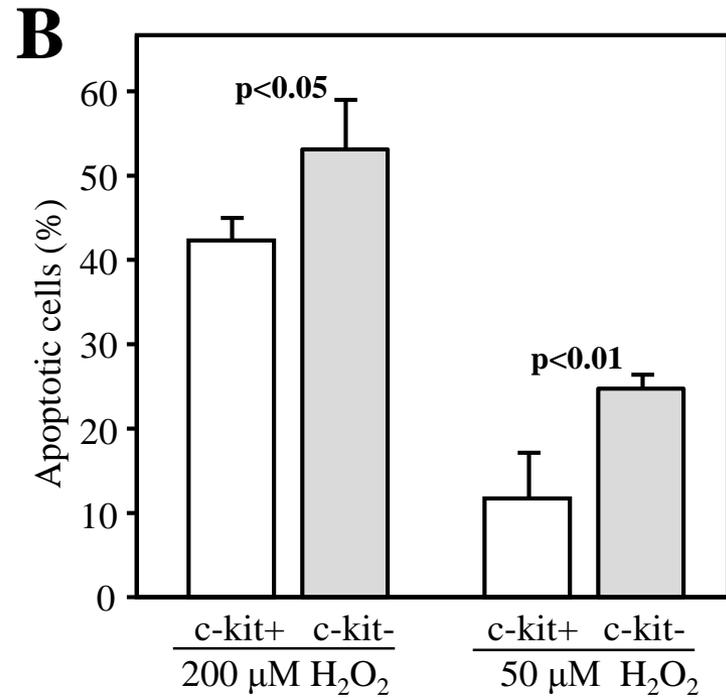
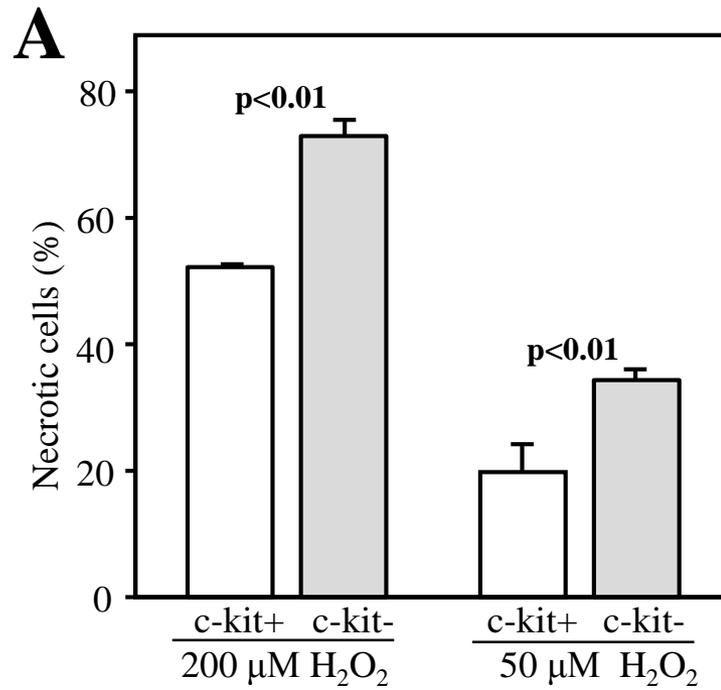


Fig 3.

A

Symbol	Fold Regulation
Nox1	10.147
Mpp4	8.151
Txnip	6.998
Ngb	4.773
Noxa1	4.708
Ptgs2	3.964
Xirp1	2.663
Il19	2.663
Nox4	2.663
Noxo1	2.608
Gpx8	-1.728
Gstk1	-1.740
Fmo2	-1.767
Sod3	-2.700
Tpo	-2.768
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Duox1	-3.315
Prdx1	-3.756
Ehd2	-4.231
Lpo	-32.424

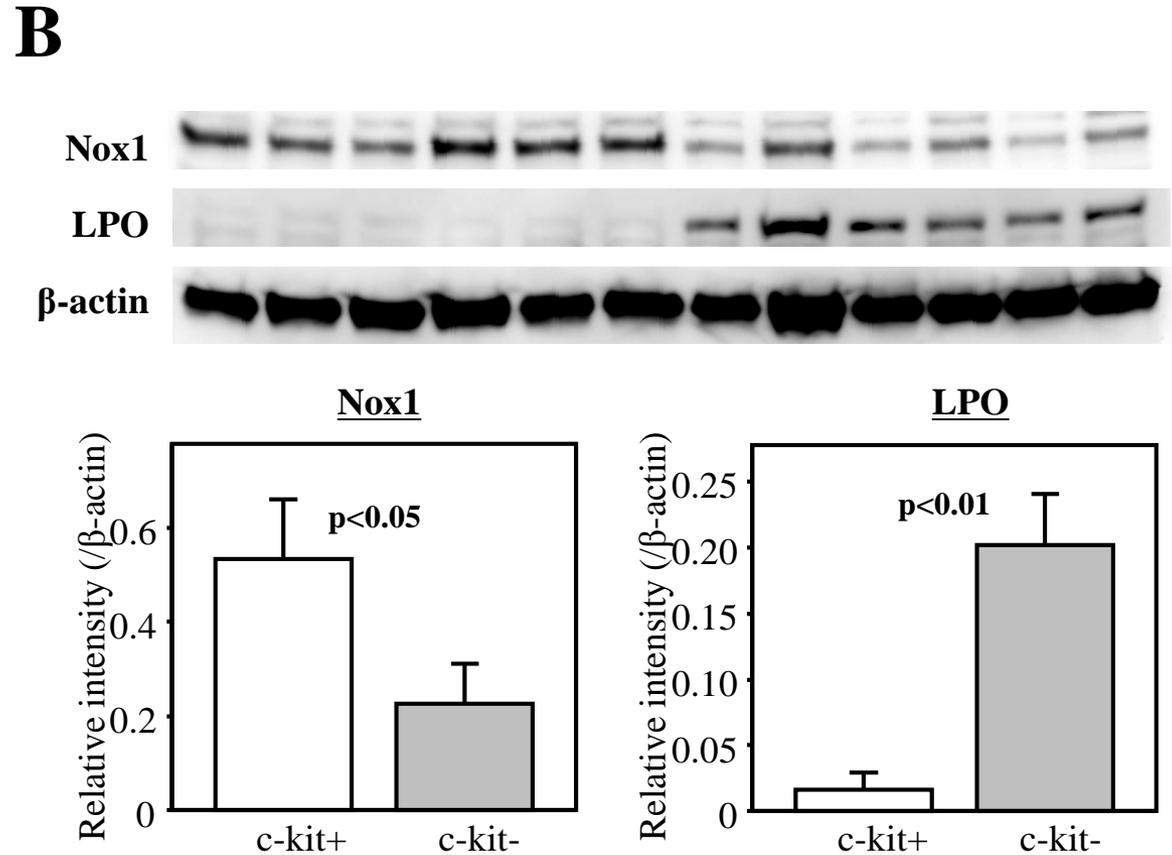
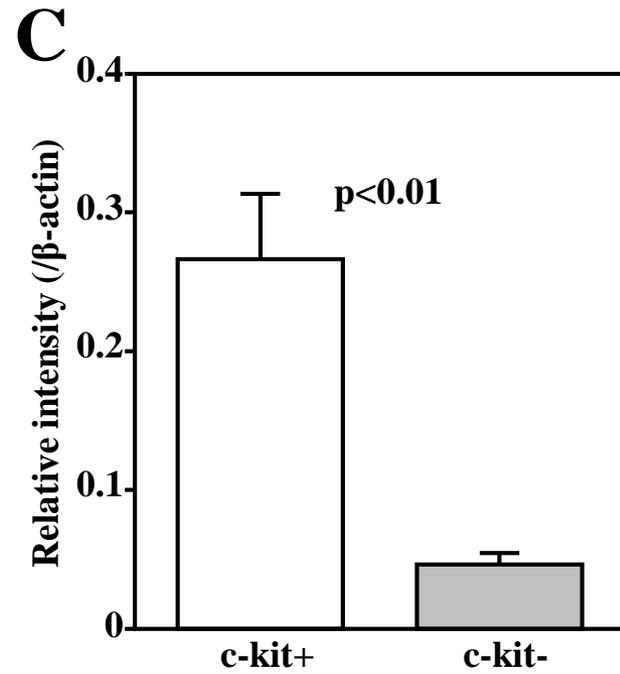
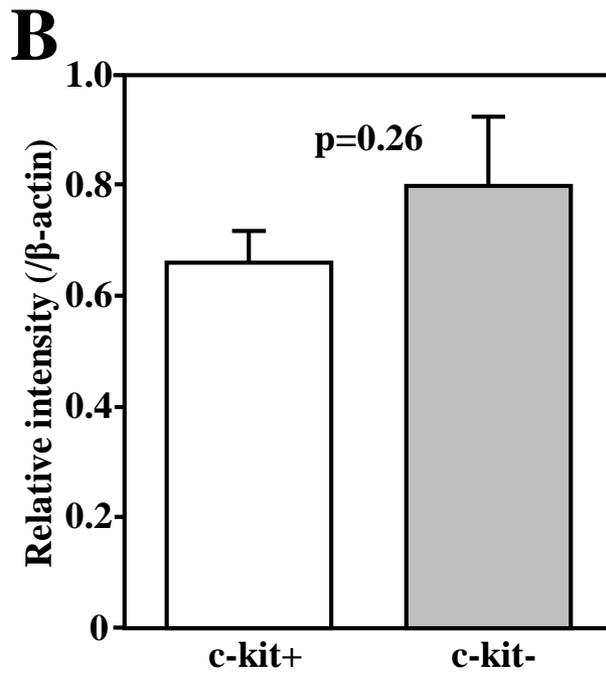
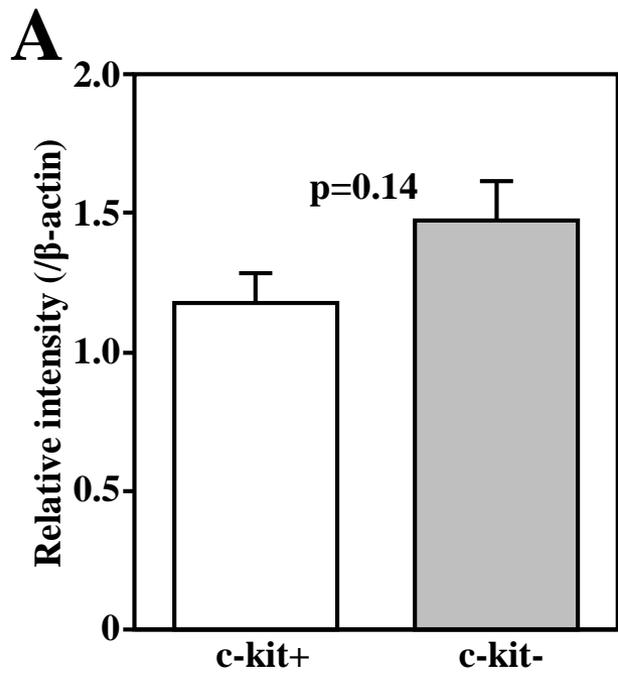


Fig 4.



Supporting information table 1. The expression of genes involved in antioxidants and ROS metabolism in the c-kit-positive hematopoietic stem/progenitor cells. (versus to the c-kit-negative matured mononuclear cells)

Symbol	Description	Fold Regulation	p value
Gpx8	Glutathione peroxidase 8 (putative)	-1.7279	0.537659
Aass	Aminoacidase-semialdehyde synthase	-1.0119	0.553957
Als2	Amyotrophic lateral sclerosis 2 (juvenile) homolog (human)	1.5105	0.331765
Apc	Adenomatosis polyposis coli	-1.1591	0.78127
Apoe	Apolipoprotein E	-1.1196	0.854769
Aqr	Aquarius	1.2354	0.621664
Atr	Ataxia telangiectasia and rad3 related	-1.2737	0.576012
Cat	Catalase	1.3426	0.798307
Ccs	Copper chaperone for superoxide dismutase	-1.0119	0.658527
Xirp1	Xin actin-binding repeat containing 1	2.6629	0.024872
Ctsb	Cathepsin B	1.232	0.763823
Cyba	Cytochrome b-245, alpha polypeptide	1.1884	0.762498
Cygb	Cytoglobin	2.5368	0.031982
Dnm2	Dynamin 2	-1.1867	0.716822
Duox1	Dual oxidase 1	-3.315	0.158261
Ehd2	EH-domain containing 2	-4.231	0.152518
Epx	Eosinophil peroxidase	1.3223	0.436183
Erc2	Excision repair cross-complementing rodent repair deficiency, complementation group 2	1.7691	0.351231
Erc6	Excision repair cross-complementing rodent repair deficiency, complementation group 6	-1.2968	0.584172
Fancc	Fanconi anemia, complementation group C	1.089	0.904312
Fmo2	Flavin containing monooxygenase 2	-1.7666	0.624024
Gab1	Growth factor receptor bound protein 2-associated protein 1	1.8213	0.353137
Gpx1	Glutathione peroxidase 1	1.3519	0.561085
Gpx2	Glutathione peroxidase 2	1.4938	0.33233
Gpx3	Glutathione peroxidase 3	-1.59	0.562552
Gpx4	Glutathione peroxidase 4	-1.5659	0.209898
Gpx5	Glutathione peroxidase 5	1.5681	0.873103
Gpx6	Glutathione peroxidase 6	1.1917	0.454657
Gpx7	Glutathione peroxidase 7	1.1196	0.42285
Gsr	Glutathione reductase	1.8391	0.090625
Gstk1	Glutathione S-transferase kappa 1	-1.7399	0.771422
Hbq1a	Hemoglobin, theta 1A	1.0432	0.799041
Idh1	Isocitrate dehydrogenase 1 (NADP+), soluble	-1.3095	0.330409
Ift172	Intraflagellar transport 172 homolog (Chlamydomonas)	1.6713	0.093488
Il19	Interleukin 19	2.6629	0.024872
Il22	Interleukin 22	1.459	0.519359
Kif9	Kinesin family member 9	-1.4369	0.915931
Lpo	Lactoperoxidase	-32.4242	0.028386
Mb	Myoglobin	1.2719	0.963656
Mpo	Myeloperoxidase	1.3407	0.546767
Mpp4	Membrane protein, palmitoylated 4 (MAGUK p55 subfamily member 4)	8.1511	0.22942
Ncf2	Neutrophil cytosolic factor 2	1.2457	0.439396
Ngb	Neuroglobin	4.7733	0.361269
Nos2	Nitric oxide synthase 2, inducible	1.2201	0.533308
Nox1	NADPH oxidase 1	10.1471	0.258432
Nox4	NADPH oxidase 4	2.6629	0.024872
Noxa1	NADPH oxidase activator 1	4.7076	0.346893
Noxo1	NADPH oxidase organizer 1	2.6081	0.065078
Nqo1	NAD(P)H dehydrogenase, quinone 1	1.6783	0.21507
Nudt15	Nudix (nucleoside diphosphate linked moiety X)-type motif 15	1.1672	0.640345
Nxn	Nucleoredoxin	-1.3278	0.311265

Park7	Parkinson disease (autosomal recessive, early onset) 7	1.457	0.438266
Ppp1r15b	Protein phosphatase 1, regulatory (inhibitor) subunit 15b	1.3315	0.153989
Prdx1	Peroxiredoxin 1	-3.7555	0.717836
Prdx2	Peroxiredoxin 2	1.2772	0.380663
Prdx3	Peroxiredoxin 3	1.0607	0.637005
Prdx4	Peroxiredoxin 4	-1.35	0.958124
Prdx5	Peroxiredoxin 5	1.5252	0.216977
Prdx6	Peroxiredoxin 6	1.244	0.605289
Prdx6-ps1	Peroxiredoxin 6, pseudogene 1	1.9386	0.111394
Prnp	Prion protein	-1.4132	0.657341
Psmb5	Proteasome (prosome, macropain) subunit, beta type 5	-1.0519	0.864667
Ptgs1	Prostaglandin-endoperoxide synthase 1	1.6923	0.444438
Ptgs2	Prostaglandin-endoperoxide synthase 2	3.9641	0.744237
Rag2	Recombination activating gene 2	-3.1016	0.31582
Recql4	RecQ protein-like 4	1.21	0.591574
Scd1	Stearoyl-Coenzyme A desaturase 1	1.0905	0.832077
Serpib1b	Serine (or cysteine) peptidase inhibitor, clade B, member 1b	1.0403	0.684418
Slc38a1	Solute carrier family 38, member 1	1.2406	0.545556
Slc41a3	Solute carrier family 41, member 3	-1.2066	0.778015
Sod1	Superoxide dismutase 1, soluble	1.2033	0.316591
Sod2	Superoxide dismutase 2, mitochondrial	-1.0622	0.703126
Sod3	Superoxide dismutase 3, extracellular	-2.7001	0.1568
Srxn1	Sulfiredoxin 1 homolog (S. cerevisiae)	-1.215	0.649599
Tmod1	Tropomodulin 1	1.1623	0.500341
Tpo	Thyroid peroxidase	-2.7683	0.244458
Txnip	Thioredoxin interacting protein	6.9983	0.754999
Txnrd1	Thioredoxin reductase 1	-1.3651	0.499423
Txnrd2	Thioredoxin reductase 2	1.1416	0.471114
Txnrd3	Thioredoxin reductase 3	1.9225	0.530422
Ucp3	Uncoupling protein 3 (mitochondrial, proton carrier)	-1.3822	0.221578
Vim	Vimentin	-1.2509	0.388304
Xpa	Xeroderma pigmentosum, complementation group A	1.9413	0.074048
Zmynd17	Zinc finger, MYND domain containing 17	1.6575	0.183099