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14. ABSTRACT

Although the causes of bone marrow failure are complicated and not completely understood, oxidative stress is recognized as a key contributor to bone marrow failure. Oxidative stress shortens the life-span of hematopoietic stem cells leading to bone marrow failure. Among the anti-oxidant enzymes that defend cells against oxidative stress, glutaredoxin (Grx) is a small redox molecule that senses oxidative stress. However, the role of Grx in bone marrow failure remains an entirely unexplored area of study. To understand the role of Grx in the regulation of hematopoietic progenitor cell function and bone marrow failure, we used Grx KO mice as our model system. The role of Grx in regulating hematopoietic progenitor cells was examined. Our results showed that Grx is important for the maintenance of hematopoietic progenitor cells in response to oxidative stress. Genetic depletion of Grx interrupts hematopoietic linage differentiation. In addition, p38 phosphorylation was significantly increased in response to hydrogen peroxide Consistent with these findings, IL-6 and Bcl-xL expression is decreased when Grx is depleted. Our results support a protective role for Grx in the prevention of bone marrow failure. Because activation of p38 disrupts the homeostasis of hematopoietic stem cells, shortens their life span, and eventually exhausts hematopoietic stem cell pools in the bone marrow leading to bone marrow failure, Grx is likely preventing bone marrow failure at least through p38 and IL-6 pathway.

15. SUBJECT TERMS
glutaredoxin, p38, hematopoietic stem cells, bone marrow failure

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Introduction:

Bone marrow failure is a group of hematopoietic stem cell disorders that affect one or more lineages of blood cells. Although the causes for bone marrow failure are complicated and not completely understood, oxidative stress has been recognized as a key contributor [1] [2] [3] [4] [5] [6]. Hematopoietic stem cells are highly sensitive to reactive oxygen species (ROS) [7, 8]. Accumulation of ROS activates p38, shortens the life span of hematopoietic stem cells, disrupts the homeostasis of hematopoietic stem cells, and eventually causes bone marrow failure [7, 8] [9] [10]. The key role of p38 in the development of bone marrow failure has also been demonstrated by the finding that treating aplastic anemia patients with a p38 MAPK inhibitor can restore defective hematopoietic activity, suggesting the critical role of p38 in bone marrow failure [11].

Regulation of hematopoietic stem cell self-renewal is essential to hematopoietic homeostasis. Interleukin-6 (IL-6) is a key regulator of hematopoiesis. IL-6 shifts hematopoietic stem cells from the G0 to the G1 stage of the cell cycle, making them more responsive to other cytokines in hematopoiesis [12]. It also stimulates the proliferation of multiple lineages of hematopoietic progenitor cells in mice and accelerates multi-lineage hematopoietic regeneration following radiation-induced hematopoietic depression [13]. Furthermore, IL-6 is involved in lineage commitment and maturation [14-17] [18, 19]. The genetic deletion of IL-6 in mice leads to aberrant production of both

immature and committed progenitors from multiple lineages in the bone marrow [16]. Thus, IL-6 regulates hematopoietic stem cell proliferation and differentiation.

Among the antioxidant enzymes that defend cell against oxidative stress, glutaredoxin (Grx) is a small redox molecule that senses hydrogen peroxide and inhibits p38. Grx binds to the C-terminus of apoptotic signaling kinase 1 (ASK1) and inhibits ASK1 from oxidative stress-induced activation [20] [21]. Grx are also involved in many other cellular processes such as resistance against oxidative stress, DNA synthesis, sulfur assimilation, apoptosis, and cellular differentiation [22] [23, 24] [25-27]. However, the role of Grx in hematopoietic stem cells and bone marrow failure is a completely unexplored area of study.

Using Grx KO mice as the model system, we studied the role of Grx in hematopoietic stem/progenitor cells and bone marrow failure. Our studies focus on the following two specific aims. Aim 1: To determine the role of Grx in regulating hematopoietic stem cell survival ex vivo. Aim 2: To determine the function of Grx in bone marrow hematopoietic stem cells in vivo.

Body:

We first established protocols for the isolation and characterization of hematopoietic stem/progenitor cells. Then we compared the ratio of KSL cells between Grx KO and wild type mice in normal condition. Bone marrow cells were isolated from both wild type and Grx KO mice followed by intracellular staining was performed using antibodies stain for hematopoietic stem cell and progenitor

cell markers (c-kit, ScaI and Lin-). The ratio of KSL cells were determined by FACS sorting (Figure 1). Under basal condition, there is no significant difference on the ratio of KSL cells between 9-10 weeks old wild type and Grx KO mice (Figure 2).

Hematopoietic stem cells are highly sensitive to oxidative stress [7, 8]. ROS disrupts the homeostasis of hematopoietic stem cells, shortens the life span of hematopoietic stem cells, and eventually exhausts hematopoietic stem cell pools in the bone marrow leading to bone marrow failure [7, 8] [9] [10].

We studies the role of Grx in ROS-induced the bone marrow failure. 9-10 weeks old wild type and Grx KO mice were treated with diquat to increases ROS, and then blood counts were performed in both wild type and Grx KO mice with and without diquat treatment.

There is no significant difference in the numbers of white blood cells, between wild type and Grx KO mice before treatment (Figure 3A). There is no significant change in the number of white blood cells in wild type mice before and after diquat administration. However, the number of white blood cells significantly decreased only in Grx KO mice after diquat treatment (Figure 3A). Interestingly, there is no significant change in the number of red blood cells between wild type mice and Grx KO mice before and after diquat treatment (Figure 3B).

Because bone marrow failure is characterized as the disrupted differentiation for one or more lineages of blood cells, our results strongly suggest that Grx is required for white blood cell linage commitment, but Grx has no influence on red blood cell linage in response to oxidative stress.

Hematopoietic stem cell self-renewal is necessary for blood cell production throughout life. It has been demonstrated that ROS causes bone marrow failure through its activation of p38. Activation of p38 shortens the life span of hematopoietic stem cells, and eventually leading to bone marrow failure [7, 8] [9] [10]. On the other hand, treating aplastic anemia patients with a p38 MAPK inhibitor can restore defective hematopoietic activity, suggesting the critical role of p38 in bone marrow failure [11].

We further tested our hypothesis that Grx depletion increases p38 activation in response to oxidative stress because Grx has been shown binding to and inhibited the upstream kinase of p38 ASK1. KSL cells isolated from 9-10 weeks old wild type and Grx KO mice were stimulated with 500 μM hydrogen peroxide for 0, 15 and 30 minutes. Intracellular staining was performed using phospho-p38 antibody. Then p38 phosphorylation was examined by phospho-flow analysis. Our results showed that even in basal level, Grx depletion had ~6-fold higher levels of phospho-p38 than in wild type mice. Moreover, p38 phosphorylation in Grx KO KSL cells was dramatically increased by hydrogen peroxide treatment (Figure 4). Because p38 activation directly contributes to the damage of hematopoietic stem cells, our results strongly suggest that Grx is likely required in the prevention of ROS-induced bone marrow failure.

Interleukin-6 (IL-6) is a key regulator of hematopoiesis. It has been demonstrated that genetic deletion of IL-6 in mice leads to aberrant production of both immature and committed progenitors from multiple lineages in the bone marrow [16]. We hypothesized that Grx modulates IL-6 pathway. It has been

demonstrated that IL-6 increases Grx protein levels, and Grx overexpression in turn increases IL-6 levels, suggesting a positive feedback loop between Grx and IL-6 [28, 29]. More importantly, Grx but not thioredoxin (Trx) expression increases during monocyte differentiation in response to IL-6 [29].

Therefore, we examined whether Grx regulates IL-6-and IL-6 pathway. RNAs were isolated from KSL cells from both wild type mice and Grx KO mice treated with either diquat or vehicle control. Quantitative PCR was performed to determine gene expressions on IL-6, and Bcl-xL in both wild type and Grx KO KSL cells using the primers listed in Table 1. Our results showed that Grx depletion dramatically decreased IL-6 and Bcl-xL levels in both control and diquat administrated mice (Figure 5).

Figures and tables

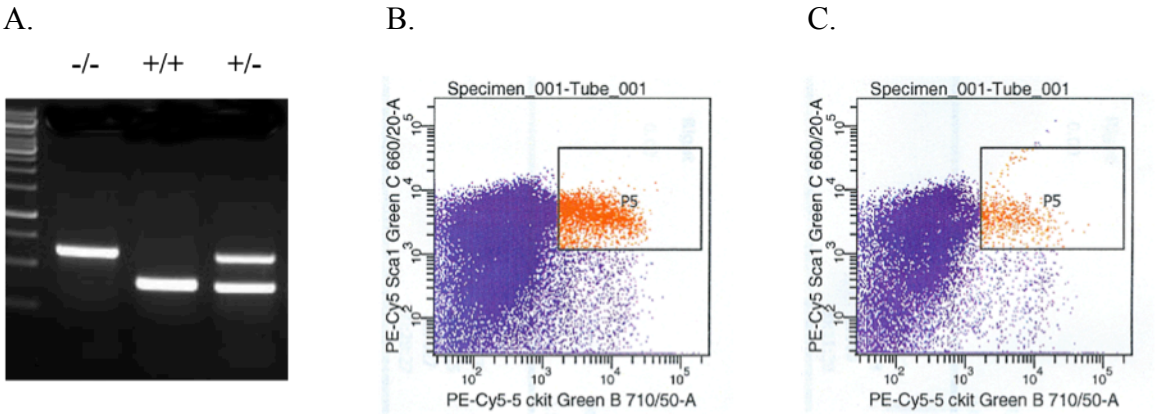


Figure 1. Genotyping of Grx KO mice (A) and cell sorting after bone marrow cells isolation (B-C).

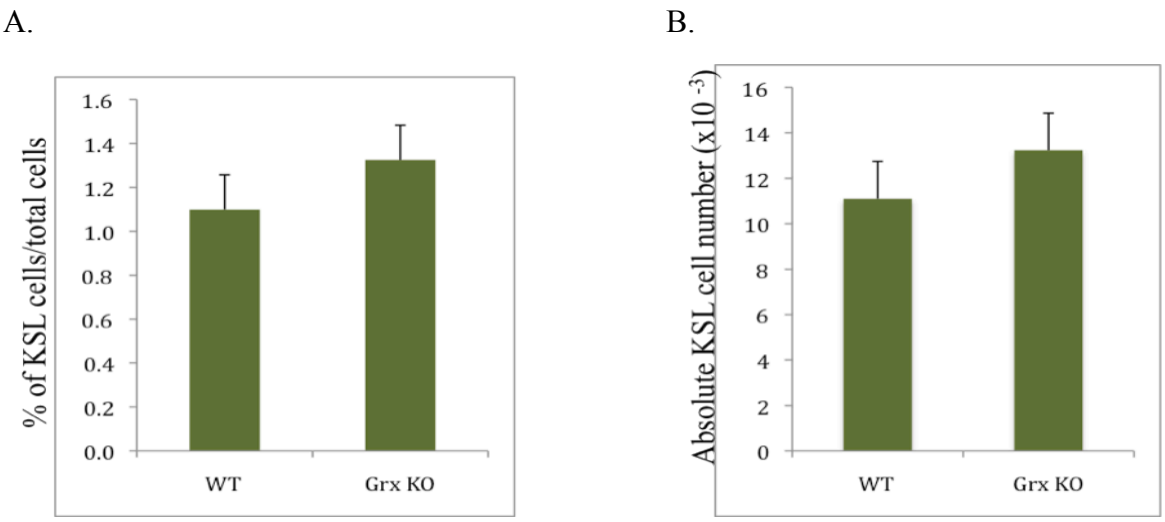
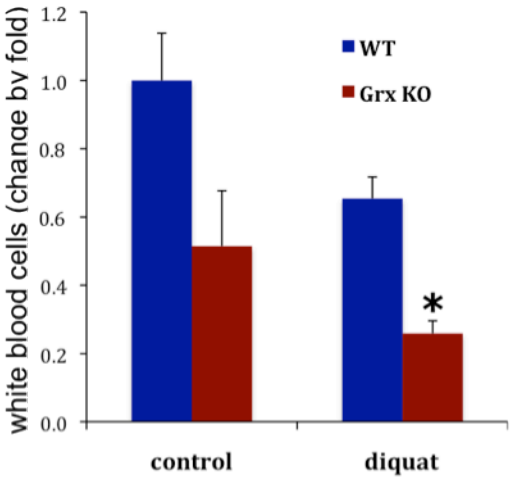


Figure 2. Ratio of isolated KSL cells from Grx KO mice and wild type littermate control mice (WT). Data are from three representative isolations and presented as percentage of KSL cells/total cells (A) and absolute KSL cell number ($x10^3$). N=3.
P> 0.05 (WT vs Grx KO) for both A. and B.

A.



B.

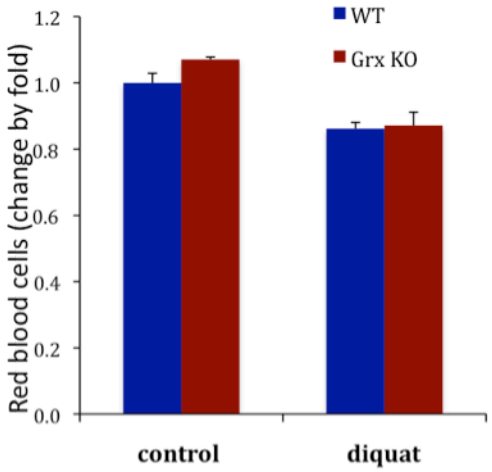


Figure 3. Blood counts in response to oxidative stress. Both wild type (WT) and Grx KO mice were treated with diquat (50mg/kg) for 72 hours. A. the number of white blood cells is significantly decreased in diquat-treated Grx KO mice. N=4, *p<0.05 vs non treatment control; B. effect of diquat administration on red blood cell count in both WT and Grx KO mice. N=4, P>0.05 vs WT control; p<0.05 vs Grx KO control.

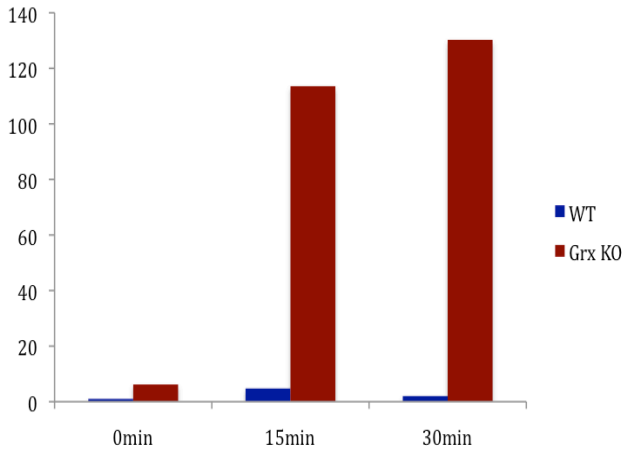


Figure 4. p38 phosphorylation in wild type (WT) and Grx KO KSL cells. KSL cells were stained with phospho-p38 antibody after hydrogen peroxide treatment (500 μM) for 0, 15 and 30 minutes. p38 phosphorylation was analyzed by setting the value of non-stimulated WT as 1.0.

A. B.

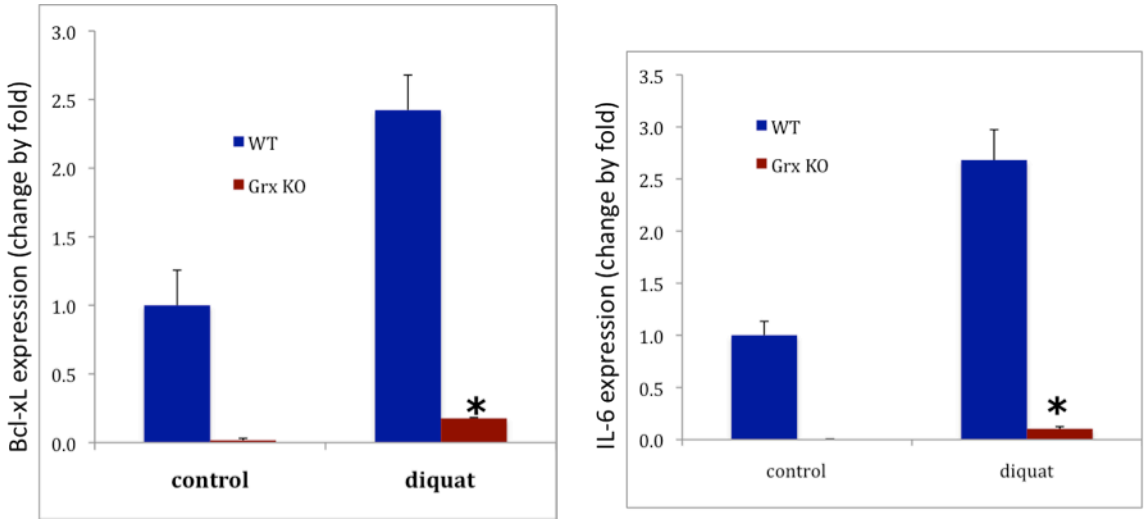


Figure 5. Expression of IL-6 (A) and Bcl-xL(B) in wild type (WT) and Grx KO mice. WT and Grx KO mice were untreated or treated with diquat for 72 hours. RNA was prepared using KSL cells isolated from WT and Grx KO mice. Expression of IL-6 and Bcl-xL were determined by quantitative PCR analysis using 18S RNA as the internal control. The results were normalized to non-treatment WT control, n=3.

Table 1. Primers for Q-PCR detection of gene expression.

IL-6	forward 5'-gac aac ttt ggc att gtg g-3' reverse 5'-atg cag gga tga tgt tct g-3'
Bcl-xL	forward 5'-agg cag gcg atg agt ttg aac-3' reverse 5'-gaa cca cac cag cca cag tca-3'

Key research accomplishments:

- Established protocol for KSL isolation from both wild type and Grx KO mice bone marrow (Figure 1).
- Examined ratio of KSL population in wild type and Grx KO mice. There is no significant difference in KSL ratio in basal level between wild type and Grx KO mice (Figure 2).
- Demonstrated the protective role of Grx in ROS-induced bone marrow failure. Examined blood component in both wild type and Grx KO mice both at the basal level and after diquat treatment. The number of white blood cells significantly decreased only in Grx KO mice after diquat treatment (Figure 3A). Interestingly, there is no significant change in the number of red blood cells between wild type mice and Grx KO mice before and after diquat treatment (Figure 3B).
- Evaluate the effect of Grx in p38 activation in bone marrow derived KSL cells. Assayed p38 activation in response to oxidative stress. There was dramatically increased p38 phosphorylation in Grx KO mice as determined by the state-of-art technique, phospho-flow analysis. Please note, there is ~6-fold increases even in the basal level of phospho-p38 in Grx KO mice (Figure 4).
- Established the critical link between Grx and IL-6 in hematopoietic homeostasis. Our results also confirmed a positive feedback loop between

Grx and IL-6. Grx depletion dramatically decreased IL-6 and Bcl-xL levels in both control and diquat administrated mice suggesting that Grx regulates IL-6-and Bcl-xL expression (Figure 5).

Conclusions:

Our results support a potentially very critical role for Grx in the prevention of oxidative stress-induced hematopoietic dysfunction and bone marrow failure.

Although in basal level there is no significant difference in the ratio of KSL cell, red blood cell and white blood cell blood counts, in response to oxidative stress the number of white blood cells were significant decreased in Grx KO mice. Because bone marrow failure is a group of hematopoietic stem cell disorders that affect one or more lineages of blood cells, our results strongly suggest that Grx may play a protective role in oxidative stress-induced bone marrow failure. Moreover, it appears that Grx is more crucial for the linage commitment of white blood cells but not red blood cells.

We found that that Grx depletion increased p38 activation in hematopoietic stem cells. The finding is potentially extremely important because increased p38 activation is found in ROS-induced bone marrow failure. Activation of p38 disrupts the homeostasis of hematopoietic stem cells, shortens their life span, and eventually exhausts hematopoietic stem cell pools in the bone marrow.

We have also demonstrated that Grx may be a key player in the regulation of hematopoietic stem cell self-renewal, which is essential to hematopoietic

homeostasis. Our q-PCR data indicate a critically link between Grx and IL-6 pathway. Grx depletion decreased IL-6 expression concomitants with down-regulation of Bcl-xL suggesting Grx may also influence hematopoietic cell homeostasis through IL-6 pathway. Our results are consistent with the finding that IL-6 plays critical role in hematopoietic homeostasis [16] and further suggest that Grx could be a potential regulator in the process.

We have demonstrated that Grx is likely to be a key player in the protection of bone marrow failure through both inhibition of p38 activation and maintain functional IL-6 pathway. Further studies such as bone marrow transplantation using Grx KO or transgenic mice will provide direct evidence on the indispensible role of Grx in hematopoietic linage commitment and bone marrow failure. More importantly, these studies may help develop new strategies for the treatment of bone marrow failure.

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