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RESVERATROL ACCELERATES ERYTHROID MATURATION BY ACTIVATION OF FOXO3 AND AMELIORATES ANEMIA IN BETA-THALASSEMIC MICE

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Running title: Resveratrol reduces ineffective erythropoiesis

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ABSTRACT
Resveratrol, a polyphenolic-stilbene, has received increased attention in the last decade due to its wide range of biological activities. β-thalassemias are worldwide distributed inherited red cell disorders characterized by ineffective-erythropoiesis and red cell oxidative damage with reduced survival. Here, we evaluated the effects of low-dose-resveratrol (5μM) on in vitro human erythroid differentiation of CD34+ from normal and β-thalassemic subjects. We found that resveratrol induces accelerated erythroid-maturation, resulting in the reduction of colony-forming-units of erythroid cells and increased intermediate and late erythroblasts. In sorted colony-forming-units of erythroid cells resveratrol activates Forkhead-box-class-O3, decreases Akt activity and up-regulates antioxidant enzymes as catalase. In an in vivo murine model for β-thalassemia resveratrol (2.4 mg/Kg) reduces ineffective-erythropoiesis, increases hemoglobin-levels, reduces reticulocyte count and ameliorates red cell survival. In both wild-type and β-thalassemic mice resveratrol up-regulates scavenging enzymes such as catalase and peroxiredoxin-2 through Forkhead-box-class-O3 activation. These data indicate that resveratrol inhibits Akt resulting in FoxO3 activation with up-regulation of cytoprotective systems enabling the pathological erythroid-precursors to resist the oxidative-damage and continue to differentiate. Our data suggest that the dual effect of resveratrol on erythropoiesis through activation of FoXO3 transcriptional factor combined with the amelioration of oxidative stress in circulating red cells may be considered as a potential novel therapeutic strategy in treating β-thalassemia.
INTRODUCTION

Resveratrol, a polyphenolic-stilbene, has received increasing attention in the last decade due to its wide range of biological activities, which include antioxidant, anti-inflammatory and anti-tumoral effects (1-3). Although some progress has been made in the identification of the mechanism(s) underlying the various beneficial effects of resveratrol, much still remains to be investigated (3, 4). Most of the studies carried out to date in evaluating the effects of resveratrol on erythropoiesis have been performed using K562 erythroleukemia cell lines (5-7) and as such very limited information is available regarding the effect of resveratrol on normal erythropoiesis (8). In these studies resveratrol has been shown to increase fetal hemoglobin synthesis (50 μM resveratrol) (6, 9), to attenuate the TNF-α effects on erythropoiesis (0.4 μM, 10 -30 μM resveratrol) (8) and to block cell growth affecting cell cycle and redirecting cells towards either apoptosis or differentiation (60 μM resveratrol) (5-7). Recently, the beneficial effects of resveratrol supplementation on pathological erythropoiesis have been reported in a mouse model for Fanconi anemia (FA), which is characterized by the hypersensitivity of FA cells to reactive oxygen species (ROS) (10). The dynamic process of erythroid differentiation is characterized by the production of reactive oxygen species (ROS) both in response to erythropoietin signaling and to the large amount of iron imported into the cells during heme biosynthesis (11). The intracellular response to oxidative-stress in erythropoiesis involves the transcription factor, Forkhead box O3a (FOXO3), which controls pathway(s) regulating erythroid maturation and the levels of oxidative stress in murine erythropoiesis (12, 13). FOXO3a is negatively regulated by the serine-threonine kinase Akt, which phosphorylates FOXO3a promoting its translocation from the nucleus to the cytoplasm and resulting in inhibition of FOXO3 transcriptional activity (12-14). Activation of FoxO3a has been proposed as a protective mechanism in pathological erythropoiesis characterized by abnormal ROS levels such as β-thalassemia (12).

β-thalassemias (β-thal) are common inherited red cell disorders characterized by absent or reduced synthesis of β-globin chains. Despite extensive knowledge of the molecular defects causing β-thalassemia, less is known about the mechanisms responsible for the associated ineffective erythropoiesis and reduced red cell survival (11, 15-20). Increased levels of reactive oxygen species (ROS) have been reported to contribute to the anemia of β- thalassemia, although the effects of ROS have not been fully defined (11, 15-18). Exogenous anti-oxidant molecules might represent complementary therapeutic strategies to counteract the toxic effects of ROS in β- thalassemia; however, few of them have been shown to beneficially affect in vivo β- thalassemic red cell features and/or thalassemic ineffective erythropoiesis in vivo (16, 21).
METHODS

In vitro erythropoiesis from CD34+ cells from peripheral circulation of normal and β-thalassemia-intermedia subjects

Cell culture, phenotypic analysis and cell sorting strategy. Peripheral blood from adult normal volunteers and from transfusion independent β-thalassemia patients (β-thalassemia intermedia) was collected, after obtaining informed consent according to the guidelines established by the Ethic Committee for human subject studies of the University of Milan and the principles of Declaration of Helsinki. Approval by the Ethic Committee of the University of Milan for human erythroid precursors studies was obtained. We analyzed 20 erythroid cultures from the peripheral blood of different normal subjects and 20 erythroid cultures from 10 homozygous β-thalassemic intermedia patients (β0cod39) (11, 17). Details on cell cultures are reported in the Supplementary Materials and Methods. The Resveratrol concentration (5 μM; Sigma Aldrich, St. Louis, MO, USA) used in this study was selected from dose-response studies (Fig. 1Sa) and a review of the literature (3, 5, 6, 8-10, 22). The erythroid cell antigen profile and the sorting of erythroid precursors were carried out as reported by Merry-Weather Clarke et al. (23). Details are reported in the Supplemental Material and Methods (Fig. 1Sb).

Quantitative-Real-time-PCR was carried out as previously described (24). Details are reported in the Supplemental Materials and Methods. Table 1S and 2S outline the details on the primers used.

Immunoblot-analysis of sorted human erythroid precursors and immunofluorescence assay for FoxO3a. 1 x 10^6 sorted CFU-E cells from both normal and β-thalassemic were solubilized as previously described (11, 20). Details on immunoblot and immunofluorescence analysis are reported in the Supplemental Materials and Methods. Whenever indicated sorted CFU-Es were separated into cytosol and nuclear fractions as previously reported (25).

Studies on mouse models

The study was carried out in accordance with the Scientific Committee for Animal Experimentation (CIRSAL, University of Verona). C57B6/2J mice, wild-type controls (WT) and Hbbth3/+ mice were employed as models of β-thalassemia. Age and sex matched mice of 2 months age (20 gr weight) were studied. The female/male ratio in the different groups studied was 1:1. Based on previous studies on resveratrol bioavailability in vivo (26-28), the mice were placed either under resveratrol (2.5 mg/Kg incorporated into AIN-93G diet) or standard diet (AIN-93G diet). We fed 2-months-old mice with resveratrol diet for 6 months. Hematological parameters and red cell indices were determined as previously reported (16, 29-32).

Cytofluorimetric analysis of mouse bone-marrow precursors and cell sorting of murine erythroblasts. The cytofluorimetric analysis of erythroid precursors from bone-marrow of both
wild-type and β-thalassemic mice was carried out as previously described (33). Population II and III corresponding to basophilic erythroblasts and polychromatric erythroblasts (1X10^6) were sorted from bone-marrow of both mouse strains for RT-PCR and immunoblot analysis. Red cell survival was carried out using CFSE (10μM; Molecular Probe, Invitrogen) (34). Details are reported in the Supplemental Materials and Methods.

**Red cell ghost preparation** (30, 35) and **red cell membrane carbonyl groups determination** were carried out as previously reported (36). Details are reported in Supplemental Materials and Methods.

**FOXO- PRDX2 alignment analysis** is detailed in Supplemental Materials and Methods.

**Statistical analysis** was carried out as detailed in Supplemental Materials and Methods.

**RESULTS**

**Low concentration of resveratrol induces erythroid maturation during normal erythropoiesis**

We first evaluated the effects of different doses of resveratrol on the production of normal erythroid cells generated from CD34+ cells isolated from peripheral blood. We found a dose dependent decrease in cell production in agreement with earlier reports that show cellular toxic effects on differentiating cells at both moderate and high doses of resveratrol (Fig. 1Sa) (5, 6, 8). We thus chose to work with a low dose of resveratrol (5 μM) and noted that even at this dose cell proliferation was decreased as compared to untreated cells (Fig. 1a). The erythroid differentiation was assessed using a combination of three surface markers: the transferrin receptor (CD71) and Glycophorin A (GPA) and CD36 (23), which enable the identification of erythroid cells at distinct stages of maturation (Fig. 1sb, 1b). Resveratrol induced a reduction of CFU-E cells but increased Int-E at day 7 and 9 of culture (early-erythropoiesis) as well as Int-E and Late-E at day 11 and 14 of culture (late-erythropoiesis) (Fig. 1b). The accelerated maturation of erythroid cells induced by resveratrol was reflected by changes in cell morphology (right panel in Fig. 1b), by increased surface expression of GPA, reduced expression of CD71 at an earlier time compared to untreated cells (Fig. 2a) and early appearance of band 3 (Fig. 1Sc). No differences in the extent of cell apoptosis as monitored by the percentage of Annexin-V positive cells (7d: untreated 3.5±0.7 % vs resveratrol 3.1 ±0.4 %, n= 6; NS; 11d: untreated 2.25±0.1% vs Resveratrol 1.9±0.8%, n=6; NS; 13d: untreated 3.5±0.7 % vs resveratrol 3.1 ±0.4 %, n= 6; NS; 11d: untreated 2.3±0.7% vs resveratrol 2.0± 0.1%, n=6; NS;) or in the expression of levels of gamma globin mRNA were noted in sorted cell erythroid populations from cultures with and without resveratrol (9d: HBG1 untreated 6.8±2.1 vs resveratrol 9.3±0.9 n=6; NS; HBG2 untreated 8.2±2.1 vs resveratrol 8.8±0.1 n=6; NS; 13d: HBG1 untreated 19.3±0.5 vs resveratrol 17.2±2.7 n=6; NS; HBG2 untreated 19.7±0.6 vs resveratrol
19.5±0.7 *n*=6; NS; HBG1 and HBG2 relative expression on GAPDH). These data imply that, while resveratrol inhibits proliferation of erythroid progenitors, it accelerates the terminal erythroid differentiation of proerythroblasts into late stage orthochromatic-erythroblasts.

Since we recently reported that resveratrol targets the transcription factor, forkhead box O3a (FOXO3a) (4), which plays a key role in erythropoiesis (12), we evaluated FOXO3a and Akt activation in sorted CFU-E cells (37, 38).

**Resveratrol enhances the expression of FOXO3a and inhibits Akt activity in sorted CFU-E**

In sorted CFU-E cells at day 7 resveratrol up-regulates FOXO3 expression without a significant change in FOXO1 mRNA levels (Fig. 2Sa). Nuclear localization of FoxO3a was used as a surrogate assay for FoxO3a activity. In sorted CFU-E cells we found that resveratrol increased the overall expression of FoxO3a protein in the nucleus (Fig. 2b), which was confirmed by immunoblot analysis (Fig. 2c). FoxO3a function is modulated by Akt activity, which phosphorylates FoxO3a, thereby sequestrating it into the cytoplasm and blocking its transcriptional activity (12, 39, 40). We then evaluated Akt activity in CFU-E cells sorted in the presence and absence of resveratrol. We found reduced phosphorylation of Akt in resveratrol treated CFU-E obtained from cultures at day 7 compared to untreated cultures (Fig. 2d). These data suggest that resveratrol enhances FOXO3 expression in CFU-E, inhibits Akt and modulates the activity of FoxO3a, sustaining erythroid precursors during accelerated erythropoiesis (Fig. 2). The activation of FoxO3a is also supported by the finding of up-regulation of catalase, a scavenging enzyme (Fig. 2Sb), whose transcription is promoted by FoxO3a (12).

**Low concentration of resveratrol hampers cell proliferation and induces cell differentiation of human β-thalassemic-erythroid cells in vitro**

To evaluate the effect of resveratrol on disordered erythropoiesis, we chose β-thalassemia as a model since its erythroid differentiation is characterized by blocked cell maturation, oxidative stress and ineffective erythropoiesis (11, 15, 17, 41, 42). CD34+ cells derived from peripheral blood of β-thalassemia intermedia subjects were studied *in vitro* (Fig. 3Sa, 3Sb). This culture system recapitulates the ineffective erythropoiesis observed *in vivo* in β-thalassemia (11, 17). Resveratrol significantly reduced cell proliferation of β-thalassemic erythroid precursors during the early-phase of erythropoiesis (7d) (Fig. 3a), with a reduction in the percentage of CFU-E cells and an increase of Int-E cells. At late stage of erythropoiesis (14d) there was a reduction of basophilic erythroblasts (Int-E) with a concomitant increase of polychromatophilic- and orthochromatic-erythroblasts (late-E), indicating a shift toward normal maturation and erythropoiesis (Fig. 3b). The increased surface expression of GPA and reduced expression of CD71 at an earlier time of culture of β-thalassemic cells in the presence of resveratrol compared to untreated cells also imply accelerated terminal
erythroid differentiation (Fig. 3Sc). No changes in the gamma-globin-chain mRNA levels were detected in sorted β-thalassemic cells with or without resveratrol (data not shown).

Resveratrol significantly increased FOXO3 expression in β-thal CFU-E cells compared to untreated β-thalassemic cells with no effects on FOXO1 mRNA levels (Fig. 4Sa). Since the number of cells from β-thalassemic erythroid precursors is limited due to the ineffective erythropoiesis and the effect of resveratrol on FoxO3a that has been shown in healthy cells by immunofluorescence and immunoblot analysis, we studied FoxO3a localization by immunofluorescence on sorted β-thalassemic CFU-E cells. We found that FoxO3a was similarly localized in the nucleus in both treated and untreated cells, but that FoxO3 nuclear expression was increased at 11 days of culture in resveratrol treated β-thalassemic CFU-E cells compared to untreated cells (Fig. 4a). We confirmed the reduced activation of Akt in resveratrol treated β-thalassemic CFU-E similar to that observed in control CFU-Es, validating a role for resveratrol on the Akt signaling pathway (Fig. 4b). In β-thalassemic CFU-E we noted up-regulation of catalase through FoxO3a activation (Fig. 4Sb). Thus, resveratrol induces erythroid differentiation by inhibiting Akt and enhancing FoxO3 activity in normal and disordered erythropoiesis, suggesting a potential role for resveratrol in reducing ineffective erythropoiesis in β-thalassemia by up-regulation of the anti-oxidant gene as catalase.

**Resveratrol decreases ineffective erythropoiesis and anemia in a in vivo mouse model of β-thalassemia**

To evaluate the impact of resveratrol treatment in vivo we used β-thalassemic mice (Hbbth3/+), a model which resembles in severity human β-thalassemia intermedia. In resveratrol treated β-thalassemic mice we observed significant increases in hematocrit, hemoglobin levels, MCV and MCH, which were associated with a significant decrease in reticulocyte count (Table 1) and a decrease in total bilirubin (WT 0.15±0.04 vs Resv. 0.2±0.06 n=6; Hbbth3/+ 0.7±0.1 vs Resv. 0.2±0.01 mg/dL; n=6 P<0.05). β-thalassemic mice treated with resveratrol exhibited a significant reduction in spleen size, a sign of extramedullary erythropoiesis (Fig. 5Sa). Erythroid precursors were evaluated by flow-cytometric analysis using the specific markers CD44 and TER119 (Fig. 5Sb) (33). In the bone marrow of resveratrol treated wild-type mice we observed a decrease in polychromatic-erythroblasts (population III) and a decrease in orthochromatic-erythroblasts compared to untreated wild-type mice (population IV; Fig. 5a and 5Sc). In the bone marrow of β-thalassemic mice resveratrol induced a decrease in basophilic erythroblasts (pop. II) and an increase in orthochromatic-erythroblasts (pop. IV; Fig. 5a and 5Sc), suggesting that resveratrol decreases the extent of ineffective erythropoiesis of β-thalassemic mice.
Resveratrol sustains *in vivo* β-thalassemic erythropoiesis by up-regulating Foxo3 and peroxiredoxin-2

In sorted basophilic-erythroblasts as seen with human cells *in vitro* Foxo3 expression was increased in both control and β-thalassemic mice treated with resveratrol (Fig. 5b). In sorted basophilic- and polychromatic-erythroblasts we found up-regulation of catalase (Cat) in both mouse strains supplemented with resveratrol similarly to that observed in cultured human cells (Fig. 5Sd). Among the scavenging enzymes with cytoprotective function we have recently reported that peroxiredoxin-2 (Prdx2) plays a crucial role in β-thalassemic red cells and proposed a role of Prdx2 in erythropoiesis (30, 35). Up-regulation of both Foxo3 and Prdx2 have been recently described in *Drosophila* neuronal cells and in mammalian cell lines exposed to oxidative stress (43, 44). By analyzing nucleotide sequences of Prdx2 and Foxo DNA binding motifs, we found a conserved FOXO binding site in the core promoter region (-15 to -8 bp) of Prdx2. We then evaluated Prdx2 expression in sorted basophilic-erythroblasts and polychromatic-erythroblasts and found up-regulation of Prdx2 in basophilic-erythroblasts from wild-type mice and in polychromatic-erythroblasts in both mouse groups treated with resveratrol (Fig. 5b). The immunoblot analysis of Prdx2 in sorted polychromatic erythroblasts confirmed the up-regulation of Prdx2 in resveratrol treated mice (Fig. 5c). These data suggest that Foxo3a might promote Prdx2 transcription.

**Resveratrol improves red cell survival and decreases red cell membrane oxidative damage in β-thalassemic mice**

Resveratrol treatment resulted in significant hematological changes in β-thalassemic mice, which are presented in Table 1. Hct and Hb levels increased significantly along with increased MCV and MCH, while the reticulocyte count and total bilirubin significantly decreased. In resveratrol treated β-thalassemic mice red cell morphology ameliorated (Fig. 6a) and erythrocyte survival studies showed a significant improvement, whereas no significant changes were noted in treated wild-type mice (Fig. 6b). We also evaluated the extent of red cell membrane oxidative damage by quantifying carbonyl groups present on red cell membrane. At baseline markedly increased membrane oxidant damage was noted in β-thalassemic mouse red cells compared to wild-type red cells (18, 30, 45) (Fig. 6c). Resveratrol supplementation significantly reduced carbonyl groups in the red cell membrane of β-thalassemic mice compared to untreated β-thalassemic mice (Fig. 6c). No differences were noted between red cells from untreated and treated wild-type mice (Fig. 6c). Since we previously showed that Prdx2 membrane association in β-thalassemic red cell is reduced, we evaluated the Prdx2 membrane binding in red cells from resveratrol treated mice. As shown in Fig. 6d, the amount of Prdx2 associated with the membrane was significantly increased in β-thalassemic mouse red cells. These data suggest that resveratrol increases red cell life-span in...
association with a decrease in red cell membrane oxidant damage in β-thalassemic mice, which in combination with beneficial effects on ineffective erythropoiesis, reduces the severity of anemia in murine β-thalassemia.

DISCUSSION
Here we show that low dose resveratrol induces early maturation of normal erythroid precursors by activation of the FoxO3a transcriptional factor, inhibition of Akt and up-regulation of antioxidant response genes such as catalase. The effects of resveratrol on cell maturation are highly dependent on resveratrol concentration and on cell types (1, 5-8). The findings from previous studies are not directly applicable to our findings due to either the use of erythroid leukemia cell lines or study of primary erythroid cells without detailed characterization of their stage of cell differentiation (5-7).

In the present study, we observed that CFU-E cells are the most susceptible erythroid cell population to the effects of low dose resveratrol (Fig. 1, 2). Since resveratrol has no effect on the expression of erythropoietin receptors during erythroid differentiation, (8) we propose that resveratrol might hamper cell proliferation and induce cell maturation as supported by the early expression of GPA and band 3 in resveratrol treated cells and by similar observations in other cell models (7, 22). We recently showed that resveratrol targets FoxO3 a key transcriptional factor in erythropoiesis involved in up-regulation of scavenging enzymes (4, 12). We explored the possibility of resveratrol playing a pivotal role as an exogenous anti-oxidant agent and as a modulator of endogenous anti-oxidant systems. In resveratrol treated CFU-E, FoxO3 was up-regulated and became predominantly localized in the nucleus, suggesting an effect of resveratrol on FoxO3a activation. This is also supported by the resveratrol inhibition of Akt, which regulates FoxO3a translocation to the nucleus (12, 13). The resveratrol induced FoxO3a activation results in the up-regulation of antioxidant response genes such as catalase, which protects erythroid cells from the cellular stress associated with accelerated erythroid maturation. We then hypothesized that these effects of resveratrol might be relevant in the disordered erythropoiesis as seen in β-thalassemia, which is characterized by ineffective erythropoiesis and oxidative stress. Previous studies have evaluated the impact of different anti-oxidant molecules on anemia of β-thalassemia, but only few of them have been shown to ameliorate β-thalassemic ineffective erythropoiesis (16, 21, 46). In resveratrol treated β-thalassemic erythroid precursors, we once again observed a reduction of CFU-E cells with an increase in Int-Es (Fig. 3b), suggesting that resveratrol accelerates β-thalassemic cell maturation through the same mechanism found in normal erythroid precursors and sustains β-thalassemic erythropoiesis by up-regulation of anti-oxidant response systems such as catalase (Fig. 4Sb). These results prompted us to carry out in vivo studies using a murine model of β-thalassemic
to determine whether resveratrol administration could modify in vivo β-thalassemic anemia. In β-thalassemic mice, resveratrol decreased ineffective erythropoiesis as supported by the reduction in spleen size and the normalization of the pattern of β-thalassemic mouse erythroid differentiation. It is of interest to note that similar results on ineffective erythropoiesis were also obtained in the same β-thalassemic mouse model treated with Jak2 inhibitor (42). Although resveratrol has been described as not affecting the EPO pathway in normal erythroid precursors (8), we cannot exclude a possible effect of resveratrol on the up-regulated Jak2 pathway in β-thalassemic erythroid precursors (42).

The beneficial effects of resveratrol on the β-thalassemic hematological phenotype was also supported by amelioration of red cell morphology, increased red cell survival, decreased anemia as reflected by increased hemoglobin values with reduced reticulocytosis and decreased levels of total bilirubin, a marker of hemolysis. The effect of resveratrol on mature red cells is supported by the reduction of the extent of red cell membrane oxidative damage and the increased membrane association of Prdx2 (1, 2, 16, 47).

The in vivo studies confirmed our findings from in vitro model of resveratrol favoring cell maturation and up-regulation of FOXO3a in sorted basophilic erythroblasts. We found that catalase and Prdx2 are also up-regulated in this erythroid population in wild-type mice and in polychromatic erythroblasts from both mouse models. These findings suggest that Prdx2 might be an additional scavenging enzyme, whose expression may be induced by FoxO3a as supported by the presence of a FOXO consensus binding site in the promoter region of Prdx2. Thus, resveratrol may confer resistance to oxidative stress in β-thalassemic erythropoiesis and there-by reduce the extent of ineffective erythropoiesis of β-thalassemia. The dose of resveratrol chosen for the in vivo mouse β-thalassemic study attains a resveratrol plasma concentration of 2.6 μM, which is close to that obtained in human subjects treated with 25 mg resveratrol (26-28). Since resveratrol bioavailability is similar in rodents and humans, our study suggests that this polyphenol might be considered as a new possible complementary tool in the treatment of anemia of β-thalassemia based on its dual role through a novel mechanism by promoting terminal erythroid differentiation with activation of FoxO3a and the up-regulation of anti-oxidant systems as catalase and Prdx2. In addition, by elucidating the FoxO3a signaling pathways affected by resveratrol in β-thalassemia, we have identified novel opportunities for targeted pharmacological interventions with compound others than resveratrol such as specific FoxO3 activators (4).

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AUTHORSHIP AND DISCLOSURES
LDF, DAS, CB, AI and SG designed the experiments, analyzed the data and wrote the manuscript; SM, NM and XA contributed to study design and the writing of the manuscript; SSF performed the experiments and analyzed the data; AS and AM performed the experiments; PR performed the FOXO3a analysis on sorted cells, analyzed the data; MB performed the carbonyl groups measurements and FOXO3a- PRDX2 bioinformatic analysis and contributed to writing of the manuscript; LDeF performed the RT-PCR analysis and analyzed the data; JA carried out the pathological analysis. MDC supplied part of the beta-thalassemia intermedia samples, and contributed to writing the manuscript; EZ contributed to sorting experiments; all authors reviewed the manuscript.

DS is a consultant to Sirtris, a GSK company.

The Authors declare no competing financial interests.

REFERENCES


TABLE 1
EFFECT OF RESVERATROL ON HEMATOLOGICAL PARAMETERS AND RED CELL INDEX IN β THALASSEMIC (Hbb^{3th/+}) MICE

<table>
<thead>
<tr>
<th></th>
<th>WT mice</th>
<th>Hbb^{3th/+} mice</th>
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<tbody>
<tr>
<td></td>
<td>Untreated n=12</td>
<td>Resveratrol n=12</td>
</tr>
<tr>
<td></td>
<td>Untreated n=14</td>
<td>Resveratrol n=12</td>
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<tr>
<td>Hct (%)</td>
<td>46.9 ± 0.7</td>
<td>46.7 ± 0.8</td>
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<tr>
<td></td>
<td>28.8 ± 0.7°</td>
<td>31.3 ± 0.4*</td>
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<tr>
<td>Hb (g/dl)</td>
<td>14.1 ± 0.1</td>
<td>14.9 ± 0.4</td>
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<tr>
<td></td>
<td>8.3 ± 0.6°</td>
<td>10.3 ± 0.5*</td>
</tr>
<tr>
<td>MCV (fL)</td>
<td>49.9 ± 1.1</td>
<td>49.0 ± 2.9</td>
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<tr>
<td></td>
<td>34.6 ± 0.6°</td>
<td>41.6 ± 5.4*</td>
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<td>MCH (pg/cell)</td>
<td>16.2 ± 0.4</td>
<td>15.8 ± 1.0</td>
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<tr>
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<td>9.7 ± 0.06°</td>
<td>12.8 ± 2.1*</td>
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<tr>
<td>CH (%)</td>
<td>13.7 ± 0.4</td>
<td>13.1 ± 0.6</td>
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<td>9.7 ± 0.2°</td>
<td>11.4 ± 2.0</td>
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<td>RDW (%)</td>
<td>12.9 ± 0.5</td>
<td>14.9 ± 0.8</td>
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<td>33.9 ± 0.8°</td>
<td>23.7 ± 8.2*</td>
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<td>Retics (%)</td>
<td>4.95 ± 0.8</td>
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<td></td>
<td>28.3 ± 4.9°</td>
<td>12.1 ± 6.0*</td>
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<tr>
<td>MCVr (fL)</td>
<td>57.2 ± 1.2</td>
<td>52.5 ± 2.7*</td>
</tr>
<tr>
<td></td>
<td>44.0 ± 1.4°</td>
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<td>CHr (pg/cell)</td>
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<tr>
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<td>11.9 ± 0.4°</td>
<td>13.5 ± 2.3*</td>
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</tbody>
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Hct: hematocrit; Hb: hemoglobin; MCV: mean corpuscular volume; MCH: mean corpuscular hemoglobin; CH: hemoglobin concentration; RDW: red cell distribution width; Retics: reticulocytes; MCVr: mean corpuscular volume reticulocytes; CHr: reticulocyte hemoglobin concentration; *: P<0.05 compared to untreated mice; °P<0.05 compared to wild-type mice

FIGURE LEGENDS
Fig. 1. Low dose resveratrol hampers cell growth and affects the pattern of erythroid maturation in normal erythropoiesis. (a) Cell proliferation of erythroid precursors derived by in vitro liquid culture of CD34+ cells isolated from peripheral blood of normal (control cells) subjects with or without resveratrol (n=10). Arrows indicate when resveratrol 5 μM was added to the culture medium. Data are presented as means± SD; * P<0.05 compared to untreated cells. **Left panel. Cytofluorimetric analysis of maturation pattern of erythroid precursors at different times of cell culture, 7, 9, 11, and 14 days (d) using the following surface markers: CD36, glycophorin-A and CD71 (see also Supplementary Methods). This cyto-fluorimetric strategy allows the identification of the following homogenous cell populations: pro-erythroblasts (Pro-E), basophilic erythroblasts corresponding to intermediate erythroblasts (Int-E) and polychromatic and orthochromatic erythroblasts as late erythroblasts (Late- E). Data are expressed as percentages or as absolute cell counts and shown as means ± SD (n=10); * P< 0.05 compared to untreated cells (Right panel). Morphology of erythroid precursors with or without resveratrol. Cytospins were stained with May-Grünwald-Giemsa. Cells were imaged under oil at 100x magnification using a Panfluor objective with 1.30 numeric aperture on a Nikon Eclipse DS-5M camera and processed with Digital Slide (DS-L1) Nikon. One representative image from a total of 10 for each condition at the different time points is shown.

Fig. 2. Low dose resveratrol induces early erythroid maturation, activates FOXO-3 and inhibits Akt pathway (a) Upper panel. Flow cytometric analysis of expression of transmembrane glycophorin-A (GPA) and CD71 during erythropoiesis at days (d) 7, 9, 11, and 14 of culture with or without resveratrol. Note the early appearance of GPA and the early reduction of CD71 in resveratrol treated cells compared to untreated cells. One representative image from a total of 10 for each condition at the different time points is shown. Lower panel. Kinetic of GPA appearance and reduction of CD71 in resveratrol treated cells compared to untreated ones. Data are presented as means ± SD (n=10); *P< 0.05 compared to untreated cells. (b) FoxO3 immunostaining of CFU-E cells at day 7 (7d) of culture with or without resveratrol. Cells were FACS-sorted, cytopunon glass slides and immunostained with anti-FoxO3a antibody and counterstained with DAPI. The mean fluorescence was measured in the nucleus of 30 cells using Image J software. *P<0.05 compared to untreated cells (n=6). (c) Immunoblot analysis of FoxO3a on nucleus of sorted CFU-E at day 7 of culture. Histone-H3 was used as loading control. One representative gel from the other 6 with similar results is presented. **Right panel. Relative quantification of immunoreactivity of FoxO3 and Histone- H3 in sorted CFU-E cells. Data are presented as FoxO3/Histone-H3 ratio and
shown as means ± SD (n=6). *P<0.05 compared to untreated cells. (d) Western-blot (Wb) analysis of phospho-Akt (p-Akt) and Akt in sorted CFU-E cells at day 7 of culture with resveratrol (Resv) or without (control, C). Tubulin was used as protein loading control. One representative gel from the other 6 with similar results is presented. Right panel. Relative quantification of immunoreactivity of phospho-Akt (p-Akt), Akt and tubulin in sorted CFU-E cells. Data are presented as p-Akt/tubulin or Akt/tubulin ratio and shown as means ±SD (n=6); *P<0.05 compared to untreated cells.

Fig. 3. β-thalassemic erythropoiesis is affected by low dose resveratrol, which induces early erythroid maturation. (a) Cell proliferation of β-thalassemic (b-thal) erythroid precursors derived by in vitro liquid culture of CD34+ cells isolated from peripheral blood of β-thalassemic (b-thal) subjects with or without resveratrol (Resv) (n=10). Arrows indicated when resveratrol 5 μM was added to the culture medium. Data are presented as means± SD; *P<0.05 compared to untreated β-thalassemic cells. (b) Cyto-fluorimetric analysis of maturation pattern of β-thalassemic erythroid precursors at different times of cell culture, 7, and 14 days (d), using the following surface markers: CD36, glycophorin-A and CD71(23) (see also on line Methods). This cyto-fluorimetric strategy allows the identification of the following homogenous cell populations: pro-erythroblasts (Pro-E), basophilic erythroblasts corresponding to intermediate erythroblasts (Int-E) and polychromatic and orthochromatic erythroblasts as late erythroblasts (Late- E). Data are expressed as percentages or as absolute cell counts and shown as means ± SD (n=10); *P< 0.05 compared to untreated β-thalassemic cells. Right panel. Morphology of β-thalassemic (b-thal) erythroid precursors with or without resveratrol (Resv). Cytospins were stained with May-Grunwald-Giemsa. Cells were imaged under oil at 100x magnification using a Panfluor objective with 1.30 numeric aperture on a Nikon Eclipse DS-5M camera and processed with Digital Slide (DS-L1) Nikon. One representative image of the other 10 for each condition at the different time points is shown.

Fig. 4. β-thalassemic sorted CFU-E from cells cultured with low dose resveratrol results in the up-regulation and activation of FoxO3a and inhibition of Akt. (a) FoxO3 immunostaining of β-thalassemic CFU-E cells at days 7 (7d) and 11 (11d) of culture with and without resveratrol. Cells are FACS-sorted, cytopun onto glass slides, and immunostained with anti-FoxO3a antibody and counterstained with DAPI. Right panel. The mean fluorescence was measured in the nucleus of 30 cells using Image J software. Data are presented as means ±SD; *P<0.05 compared to untreated cells (n=5). (b) Western-blot (Wb) analysis of phospho-Akt (p-Akt) and Akt in sorted β-thalassemic CFU-E cells at days 7 and 11 of culture with resveratrol (Resv) or without (control, C). Tubulin was used as protein loading control. One representative gel from the other 6 with similar results is presented. Right panel. Relative quantification of immunoreactivity of of phospho-Akt (p-Akt),
Akt and tubulin in sorted CFU-E cells. Data are presented as p-Akt/tubulin or Akt/tubulin ratio and shown as means ±SD (n=6). *P<0.05 compared to untreated cells.

**Fig. 5.** *In vivo* supplementation with resveratrol ameliorates β-thalassemic ineffective erythropoiesis, up-regulates Foxo3 and peroxiredoxin-2 (Prdx2). (a) Cyto-fluorimetric analysis of maturation pattern of wild-type (wt) and β-thalassemic (b-thal) erythroid precursors from the bone marrow of mice with or without resveratrol supplementation using the following surface markers: CD44 and TER119 (see also the Supplementary Materials and Methods and Fig. 5Sb). This cyto-fluorimetric strategy allows the identification of the following homogenous cell populations: population I corresponding to pro-erythroblasts, population II corresponding to basophilic erythroblasts, population III corresponding to polychromatic erythroblasts and population IV corresponding to orthochromatic erythroblasts. Data are presented as means ± SD (absolute cell counts are shown in Fig. 5Sc) (n=10); *P< 0.05 compared to untreated mice. (b) **Left panel:** RT-PCR expression of Foxo3 and peroxiredoxin-2 (Prdx2) on sorted basophilic erythroblasts from bone marrow of mice with or without resveratrol (Resv) supplementation. Sorted basophilic erythroblasts and polychromatic erythroblasts from 10 different mice from each mouse group were analyzed. Experiments were performed in triplicate. Error bars represent the standard deviations (mean ± SD; *P <0.05 compared to untreated mice, n=10). **Right panel:** RT-PCR expression of peroxiredoxin-2 (Prdx2) on sorted polychromatic erythroblasts from the bone marrow of mice with or without resveratrol (Resv) supplementation. Sorted polychromatic erythroblasts from the 10 different mice from each group were analyzed. Experiments were performed in triplicate. Error bars represent the standard deviations (mean ± SD; *P <0.05 compared to untreated mice; n=10). (d) Immunoblot analysis of PRDX2 in sorted basophilic and polychromatic erythroblasts of wild-type (wt) and β-thalassemic (b-thal) mice with or without resveratrol (Resv) supplementation. Band 3 was used as loading control. One representative experiments of 4 others with similar results. **Right panel.** Relative quantification of immunoreactivity of peroxiredoxin-2 (Prdx2) and band 3 in sorted polychromatic erythroblasts of wild-type (wt) and β-thalassemic (b-thal) mice with or without resveratrol (Resv) supplementation. Data are expressed as Prdx2/band 3 ratio and presented as means ±SD (n=5); *P <0.05 compared to untreated mice.

**Fig. 6.** *In vivo* supplementation with resveratrol ameliorates red cell morphology, increases red cell lifespan and reduces red cell membrane oxidative damage in β-thalassemic mice. (a) Morphology of red cells from wild-type (wt) and β-thalassemic (b-thal) mice with or without resveratrol supplementation. Cells were stained with May-Grunwald-Giemsa. Cells were imaged
under oil at 100x magnification using a Panfluor objective with 1.30 numeric aperture on a Nikon Eclipse DS-5M camera and processed with Digital Slide (DS-L1) Nikon. We show one representative image from a total of 12 for each condition. (b) Red cell survival (see also Methods) of CFSE labeled red cells from wild-type (wt) and β-thalassemic (b-thal) mice with or without resveratrol (Resv) supplementation. Data are presented as means ± SD (n=4) from each mouse group; *P< 0.05 compared to untreated mice. (c) Percentage of carbonyl groups from red cell membranes from wild-type (wt) and β-thalassemic (b-thal) mice with or without resveratrol (Resv) supplementation. Data are presented as means ± SD (n= 6) from each group; *P< 0.05 compared to untreated mice; °P< 0.05 compared to wild-type mice. (d) Peroxiredoxin-2 (Prdx2) membrane association in wild-type (wt) and β-thalassemic (b-thal) mice with or without resveratrol (Resv) supplementation. Actin was used as loading control protein. **Right panel.** Relative quantification of immunoreactivity of peroxiredoxin-2 (Prdx2) and actin in red cell membrane from wild-type (wt) and β-thalassemic (b-thal) mice with or without resveratrol (Resv) supplementation. Data are presented as means ±SD (n=6); *P< 0.05 compared to untreated mice.
Fig. 5

(a) Graph showing percentage of cells across different groups.

(b) Graphs comparing mRNA expression of foxt3 and Prdx2 between Wt and Wt+Resv, and b-thal and b-thal+Resv.

(c) Western blot analysis of Prdx2 and Band 3 levels in Wt and b-thal with and without Resv treatment.
SUPPLEMENTARY MATERIALS AND METHODS

Drugs and chemicals. NaCl, KCl, Na₂HPO₄, Na₃VO₄, KH₂PO₄, MgCl₂, NH₄HCO₃, MOPS, HEPES, TRIS, N-ethylmaleimide (NEM), choline chloride, benzamidine, β-mercaptoethanol, glycine, bromphenol blue, trypsin, sodium dodecil sulphate (SDS) and glycerol were obtained by Sigma/Aldrich (St Louis, MO, USA); protease inhibitor cocktail tablets were from Roche (Basel, Switzerland); Triton X-100, ECL-Plus, Percoll were purchased from GE Healthcare (Little Chalfont, UK); 40% Acrylamide/Bis Solution, 37.5:1 was from BIO-RAD (Hercules, CA, USA).

In vitro erythropoiesis from CD34⁺ cells from peripheral circulation of normal and β-thalassemia-intermedia subjects

Cell culture, phenotypic and cell sorting strategy.

Light-density mononuclear cells were obtained by centrifugation on Lymphoprep (Nycomed Pharma, Oslo, Norway) density gradient, as previously described (11, 17). The CD34⁺ cells were positively selected by anti-CD34-tagged magnetic beads (Mini-MACS columns; Miltenyi Biotech, Auburn, CA) according to the manufacturer’s protocol. The recovery was more than 90% CD34⁺ cells, as determined by flow cytometry. CD34⁺ cells were grown at a density of 10⁵ cells/mL in alpha-minimal essential medium (α-MEM; GIBCO, Grand Island, NY) supplemented with 100 U/mL penicillin-streptomycin, 2 mmol/L L-glutamine, 10⁻⁶ mol/L hydrocortisone, 10⁻³ g/L nucleotide, 25x10⁻³ mg/L gentamicin, 10⁻⁴ mol/L 2-mercaptoethanol, 1% deionized bovine serum albumin [BSA] (all from Sigma, St Louis, MO), 30% fetal bovine serum (FBS, GIBCO, Grand Island, NY), 1 µg/mL Cyclosporin A (Sigma, St Louis, MO). The following recombinant cytokines were added to the media: 3 U/mL recombinant human (rH) erythropoietin (rHuEPO, Janssen-Cilag, Milan, Italy), 20 ng/mL rH stem cell factor (SCF, PeproTech, London, UK), 10 ng/mL rH interleukin-3 (IL-3, PeproTech, London, UK). In control cell cultures, cell samples were collected at day 7, 9, 11 and 14 of culture for cell counting and determination of cell viability. In β-thalassemic cell cultures, cell samples were collected at days 7 and 14 for cell counting and determination of cell viability. Cell morphology was analyzed on cytospin smears stained with May-Grunwald-Giemsa.

Resveratrol (5 µM; Sigma Aldrich, St. Louis, MO, USA) was added at days 1 and 7 of phase 1 of the cell cultures, when a change in liquid culture medium is required to induce terminal differentiation of erythroid precursors. Then, resveratrol was added to culture media at days 9, 11 and 13 of phase 2 of the cell cultures to ensure the continuous presence of active compound during
later stages of erythroid development since its effects have been described as persisting for only 48 hours (8).

The erythroid cell antigen profile was analyzed using the recently reported cytofluorimetric strategy with the following surface markers: CD71 (anti-CD71 fluorescein isothiocyanate (FITC)-conjugated; BD Biosciences, San Jose, CA, USA), glycophoryin A (GPA, phycoerythrin (PE)-conjugated anti-CD235a; BD Biosciences, San Jose, CA, USA) and CD36 (anti-CD36, allophycocyanin (APC)-conjugated; BD Biosciences, San Jose, CA, USA) (22). All the analyses were performed with the flow cytometer FACSCanto™ (Becton Dickinson, San Jose, CA, USA). Data were stored and processed using FACSDiva software (Becton Dickinson Immunocytometry System, San Jose, CA, USA). The biparametric scatter plots were analyzed with FlowJo software version 7.6.4 (Tree Star, Ashland, OR, USA). Unstained cells were used as a negative control. CFU-E, Pro-E Int-E and Late-Erythroblasts were sorted as reported by Merry-Weather Clarke et al. using a FACSArray™ II instrument (Becton Dickinson, San Jose, CA, USA) (23). Cell apoptosis was determined on sorted CFU-E cells by double-staining the cells with FITC-conjugated Annexin-V and PI. The human Annexin-V-FITC Apoptosis Detection Kit (Bender Medsystems, Vienna, Austria) was used, according to the manufacturer’s instructions.

**Quantitative-Real time PCR.** For quantitative real time (qRT)-PCR mRNA was isolated and reverse transcribed into high-purity cDNA using μMACS One-step cDNA Kit according to the manufacturer’s instructions (Miltenyi Biotech). We started from either 250,000 sorted human CFU-E cells or sorted basophilic erythroblasts or polychromatic erythroblasts from bone marrow of both mouse strains with and without resveratrol. 1/50th of the reactions were added to appropriate wells of the PCR plates. qRT-PCR was performed by SYBR Green PCR Master Mix (Applied Biosystems) by using Applied Biosystems Model 7900HT Sequence Detection System. Detailed methods and primer sequences are available on request and Table 1S and 2S. All PCR reactions were performed in triplicate. Relative gene expression was calculated by using the $2^{-\Delta\Delta Ct}$ method, in which $Ct$ indicates cycle threshold, the fractional cycle number where the fluorescent signal reaches the detection threshold (24). The $\Delta Ct$ was computed by calculating the difference of the average $Ct$ between the X-gene and the internal control GAPDH. The data are presented as mean ± the standard deviation (SD).

**Immunofluorescence assay for FoxO3a and immunoblot analysis of FoxO3a.** 250,000 sorted CFU-E cells at day 7 were obtained from control and at days 7 and 11 from β-thalassemic cultures. Cells were cytospun onto glass slides and fixed with 4% paraformaldehyde. After washing with PBS, cells were permeabilized using 3% bovine serum albumin, 0.25% Triton X-100 and then
incubated in 1% bovine serum albumin overnight with the anti-FoxO3a (Millipore, Temecula, CA USA). Cells were washed and stained with Alexa Fluor 594 anti-rabbit IgG antibody (Molecular Probes). Nuclei were stained with Vectashield-DAPI (Vector Laboratories). Images were captured using Nikon EclipseE600 microscope. Quantification was performed using J software. Whenever indicated sorted CFU-E were separated in cytosol and nuclear fractions as previously reported (25).

**Immunoblot analysis of sorted erythroid precursors.** 1 x 10⁶ sorted CFU-E cells at 7 days of culture normal and at 7 and 11 days of culture of β-thalassemic cultures were solubilized as previously described (11, 17). Proteins were separated by monodimensional electrophoresis and transferred to membrane for immunoblot analysis with specific antibodies against Akt (Millipore, Temecula, CA USA), phospho-Akt (p-Akt, Millipore, Temecula, CA USA); FoxO3a (Millipore, Temecula, CA USA), Tubulin (University of Colorado, Boulder, CO USA) and Histone-H3 (Cell Signaling) were used as loading control. Whenever indicated erythroid population as CD36+, CD71 + cells were sorted to carry out the analysis of band 3 expression. The cells were solubilized as reported above and analyzed by immunoblot using anti-band 3 (IVF12, ML Jennings, University of Arkansas for Medical Science, USA) and anti-actin (Sigma, St Louis, MO) as loading control. We carried out the densitometric analysis of the scanned images of unsaturated films (ImageJv 1.28 software).

**Immunofluorescence assay for Foxo3a.** 250.000 sorted CFU-E at each time point for control and β-thalassemic cultures were cytospun onto glass slides and fixed with 4% paraformaldehyde. After washing with PBS, cells were permeabilized using 3% bovine serum albumin, 0.25% Triton X-100 and then incubated in 1% bovine serum albumin overnight with the anti-FoxO3a (Millipore, Temecula, CA USA). Cells were washed and stained with Alexa Fluor 594 anti-rabbit IgG antibody (Molecular Probes). Nuclei were stained with Vectashield-DAPI (Vector Laboratories). Images were captured using Nikon EclipseE600 microscope. Quantification was performed using J software.

**Studies on mouse models**

Preliminary experiments showed that low dose resveratrol has limited hematological effects on older β-thal mice or for shorter periods of treatment of 1 or 2 months. Mouse behavior and weight were monitored monthly.

**Hematological parameters and red cell indices.** Blood was collected by retro-orbital venipuncture in anesthetized mice using heparined microcapillary tubes. Hematological parameters were evaluated on a Bayer Technicon Analyser ADVIA. Hematocrit and hemoglobin were manually determined as previously described (16, 29-32).
Cytofluorimetric analysis of mouse bone marrow precursors and immunoblot analysis of sorted murine erythroblasts. The cytofluorimetric analysis of erythroid precursors from bone marrow of both wild-type and blast was carried out as previously described using CD44-FITC (eBioscience, San Diego, CA USA), TER-119 APC (eBioscience, San Diego, CA USA) strategy (24, 33). Population II and III corresponding to basophilic and polychromatic erythroblasts (1X10^6) were sorted from bone marrow of both wild-type and β-thalassemic mice with and without resveratrol supplementation as previously reported (20, 33). Cells were used for RT-PCR analysis (see above). In sorted polychromatic erythroblasts Prdx2 expression was evaluated by immunoblot analysis using the specific antibody against Prdx2 C-terminal as gently gifted by prof. Chae HZ, Chonnam National University, Korea).

Red cell survival. Red cells from wild-type and β thalassemic mice treated with or without resveratrol were labeled with CFSE (10μM; Molecular probe, Invitrogen) in PBS, BSA 0.5% fro 20 min at 37°C. After quencing with PBS in presence of FBS 1%, red cells washed 3 times with sterile PBS and resuspended at 2 x 10^9 cells in 250 μL of sterile PBS. Wild-type and β thalassemic mice were injected intravenously with the CFSE-labeled red cells, the kinetics of disappearance of labeled cells from circulation was measured by flow-cytometry (34).

Red cell ghost preparation and immunoblot analysis. Red cell ghosts from red cells of resveratrol treated and untreated mouse groups were prepared as previously described (30, 35). Whenever PRDX2 was evaluated in one-dimensional (1D) SDS-PAGE analysis, 100 mM of NEM was added to the lyses buffer to avoid possible artifacts due to PRDX2 oxidation after cell lysis. For mono-dimensional electrophoresis proteins from ghosts and cytosol fraction were solubilized in non-reducing sample buffer (SB: 50 mM Tris pH 6.8, 2% SDS, 10% glycerol, a few grains of bromphenol blue). Gels were transferred to nitrocellulose membranes for immuno-blot analysis with specific anti-Peroxiredoxin-2 antibody (AbCam, Cambridge, UK) and anti-actin (Sigma Aldrich, USA). Blots were developed using chemiluminescence reagents (ECL, Amersham).

Red cell membrane carbonyl groups. Carbonyl- groups of red cell membranes were determined as reported with slight modifications (36). Approx. 20-50 ml of membrane suspension was diluted to 1 ml with 2M HCl and treated with 40 ml of 5 mM 2,4-dinitrophenylhydrazine (2,4-DNPH) (final concentration 0.2 mM) in 2M HCl for one hour at room temperature to allow denaturation and complete reaction of carbonyl groups and their conversion into the 2,4-dinitrophenylhydrazone derivatives. Absorbance at 370 nm was then measured subtracting a blank, which contained 0.2 mM 2,4-DNPH alone.
**FOXO-PRDX2 alignment analysis.** The sequence alignment between the nucleotide sequence of the promoter region of PRDX2 and the FOXO-DNA binding motif was performed using the web available program ClustalW.

**Statistical analysis.** Data were analyzed using either *t*-test or the 2-way analysis of variance (ANOVA) algorithm for repeated measures between control and β thalassemic cultures or mice with or without resveratrol at the different time point studied. A difference with a *P* value less than 0.05 was considered significant.
Table 1S. Details of primers for the specific Q-RT PCR of the analyzed genes in sorted CFU-E

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Table 2S. Details of primers for the specific Q-RT PCR of the analyzed genes on sorted mouse basophilic erythroblasts and polychromatic erythroblasts.

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**SUPPLEMENTAL FIGURES**

**Fig. 1S.** (a) Dose response effects of resveratrol (no resveratrol: control; 1, 5, 10, 25 μM) on cell growth of human erythroid precursors derived from CD34+ cells isolated from peripheral blood of normal subjects (n=5). Data are presented as means ± SD. The arrows indicate when resveratrol was added to the medium of the cultures. (b) Flow cytometric strategy of human erythroid precursors derived from CD34+ cells analyzed using CD36, CD71 and GPA as surface markers as previously reported by Merryweather *et al.* (22). (c). Immunoblot analysis of band 3 from sorted CD71+, CD36+ erythroblasts with and without resveratrol treatment. One experiment similar to the other 4 is shown. Actin was used as a loading control protein.
Fig. 2S. (a) RT-PCR expression of FOXO3 and FOXO1 on sorted CFU-E cells at day 7 of culture with and without resveratrol. Sorted CFU-E cells from 4 different cell cultures were analyzed. Experiments were performed in triplicate. Error bars represent the standard deviations (mean ± SD; *P < 0.01 compared to untreated cells; n=4). (b) RT-PCR expression of catalase (CAT) on sorted CFU-E cells at day 7 of culture with and without resveratrol. Sorted CFU-E cells from 4 different cell cultures were analyzed. Experiments were performed in triplicate. Error bars represent the standard deviations (mean ± SD; *P < 0.01 compared to untreated cells; n=4).
**Fig. 3S.** (a) Cell proliferation of normal (control) and β-thalassemic (b-thal) erythroid precursors derived by *in vitro* liquid culture of CD34⁺ cells isolated from peripheral blood of healthy and β-thalassemic (b-thal) intermedia subjects (*n*=10). Data are presented as means± SD, *n*=10; ° *P*<0.05 compared to control cells. (b) Cyto-fluorimetric analysis of maturation pattern of normal (Control) and β-thalassemic erythroid precursors at different time of cell culture 7, and 14 days (d) using the following surface markers: CD36, glycophorin-A and CD71 (see also on line Methods). This cyto-fluorimetric strategy allows the identification of the following homogenous cell populations: pro-erythroblasts (Pro-E), basophilic erythroblasts corresponding to intermediate erythroblasts (Int-E) and polychromatic and orthochromatic erythroblasts as late erythroblasts (Late-E). Data are presented percentage and absolute cell counts and shown as means± SD (*n*=10); ° *P*< 0.05 compared to control cells. (c) Kinetic of GPA appearance and reduction of CD71 in resveratrol (Resv) treated β-thalassemic cells compared to untreated cells at 7 and 14 days of culture. Data are presented as means ± SD (*n*=10); *°* *P*< 0.05 compared to untreated cells.
Fig. 4S. (a) RT-PCR expression of FOXO3 and FOXO1 on sorted β-thalassemic (b-thal) CFU-E cells at day 7 of culture with and without resveratrol (Resv). Sorted β-thalassemic CFU-E cells from 6 different cell cultures were analyzed. Experiments were performed in triplicate. Error bars represent the standard deviations (mean ± SD, n=4; *P <0.05 compared to untreated cells). (b) RT-PCR expression of catalase (CAT) on sorted β-thalassemic CFU-E cells at day 7 and 11 of culture with and without resveratrol (Resv). Sorted CFU-E cells from 4 different cell cultures were analyzed. Experiments were performed in triplicate. Error bars represent the standard deviations (mean ± SD; n=4; *P <0.01 compared to untreated cells).
Fig. 5S. (a) Spleen weight/mouse weight ratio in wild-type (wt) and β-thalassemic (b-thal) mice with and without resveratrol supplementation. Data are presented as means ± SD (n=12); *P< 0.05 compared to untreated mice. (b) Flow cytometric strategy of mouse erythroid precursors using CD44, TER119 strategy as previously reported (31, 41). (c) Cyto-fluorimetric analysis of maturation pattern of wild-type (wt) and β-thalassemic (b-thal) erythroid precursors from bone marrow of mice with or without resveratrol supplementation using the following surface markers: CD44 and TER119 (see also supplementary Materials and Methods). This cyto-fluorimetric strategy allows the identification of the following homogenous cell populations: population I corresponding to pro-erythroblasts, population II corresponding to basophilic erythroblasts, population III corresponding to polychromatic erythroblasts and population IV corresponding to orthochromatic erythroblasts. Data are presented as absolute cell counts and shown as means ± SD (n=10); *P< 0.05 compared to untreated mice. (d) RT-PCR expression of catalase (Cat) on sorted basophilic and polychromatic erythroblasts from wild-type (WT) and β-thalassemic (b-thal) mice with or without resveratrol (Resv) supplementation. Sorted cells from 6 mice from each group were
analyzed. Experiments were performed in triplicate. Error bars represent the standard deviations (mean ± SD, n=6; *P <0.01 compared to untreated cells).