Oxidative stress modulates heme synthesis and induces peroxiredoxin-2 as a novel cytoprotective response in β-thalassemic erythropoiesis

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Citation: De Franceschi L, Bertoldi M, De Falco L, Santos Franco S, Ronzoni L, Turrini F, Colancecco A, Camaschella C, Cappellini MD, and Iolascon A. Oxidative stress modulates heme synthesis and induces peroxiredoxin-2 as a novel cytoprotective response in β-thalassemic erythropoiesis. Haematologica 2011;96(11):1595-1604. doi:10.3324/haematol.2011.043612

Supplementary Design and Methods

Drugs and chemicals

NaCl, KCl, Na_2HPO_4, Na_3VO_4, KH_2PO_4, $MgCl_2$, NH_4HCO_3, MOPS, HEPES, TRIS, N-ethylmaleimide (NEM), choline cloride, benzamidine, β-mercaptoethanol, glycine, bromophenol blue, trypsin, sodium dodecyl sulfate (SDS), chlorpromazine, hemin hydrochloride, bovine liver catalase, bovine erythrocyte superoxide dismutase, hydrogen peroxide 30% (v:v) solution, succinylcoA, glycine, thiamine pyrophosphate, α-ketoglutarate dehydrogenase, α-ketoglutarate, NAD^+, NADPH, *E. coli* thioredoxin, *E. coli* thioredoxin reductase and glycerol were obtained from Sigma/Aldrich (St Louis, MO, USA); urea, thiourea, dithiothreitol (DTT), iodoacetamide, try-n-butylphosphate, trifluor acetic acid and α-cyano-4-hydroxy cynnamic acid were from Fluka (Buchs, Switzerland); CHAPS and low melting (LM) agarose were from USB (Cleveland, OH, USA); acetone, methanol, and acetonitrile were from Baker (Deventer, the Netherlands); protease inhibitor cocktail tablets were from Roche (Basel, Switzerland); Immobiline DryStrip 7 cm pH 4-7 gels, IPG buffer pH 3-10, Triton X-100, ECL-Plus, and Percoll were purchased from GE Healthcare (Little Chalfont, UK); 40% acrylamide/bis Solution, 37.5:1 was from BIO-RAD (Hercules, CA, USA).

Cell cultures of erythroid precursors

Low-density mononuclear cells were obtained by centrifugation on a Lymphoprep (Nycomed Pharma, Oslo, Norway) density gradient, as previously described.[1-3] The $CD34^+$ cells were positively selected by anti-CD34-tagged magnetic beads (MiniMACS columns; Miltenyi Biotech, Auburn, CA, USA) 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 10^5 cells/mL in alpha-minimal essential medium (α-MEM; GIBCO, Grand Island, NY, USA) supplemented with 100 U/mL penicillin-streptomycin, 2 mmol/L L-glutamine, 10^{-6} mol/L hydrocortisone, 10^{-3} g/L nucleotide, 25×10^{-3} mg/L gentamicin, 10^{-4} mol/L 2-mercaptoethanol, 1% deionized bovine serum albumin (BSA) (all from Sigma, St Louis, MO, USA), 30% fetal bovine serum (FBS) (GIBCO, Grand Island, NY, USA), and 1 μg/mL cyclosporine A (Sigma, St Louis, MO, USA). The following recombinant cytokines were added to the media: 3 U/mL recombinant human (rHu) erythropoietin (EPO, Janssen-Cilag, Milan, Italy), 20 ng/mL rHu stem cell factor (SCF, PeproTech, London, UK), 10 ng/mL rHu interleukin-3 (IL-3, PeproTech, London, UK).

Measurements of hydrogen peroxide and superoxide anion as reactive oxygen species in erythroid precursors

Aliquots of 20-50 μL of the cytoplasmic fractions of day 7 and 14 erythroid precursors from controls or β-thalassemic patients were diluted into 20 mM potassium phosphate buffer, pH 7.4 containing 1 mM DTT in a total volume of 0.7 mL. To these mixtures, 10 μL of bovine liver catalase 25 mg/mL (C-9322, Sigma-Aldrich) or 10 μL of bovine erythrocyte superoxide dismutase solution (S7446, Sigma-Aldrich) were added at high excess to remove all H_2O_2 and O_2^- present. The enzymatically generated oxygen formation was measured amperometrically with a Clark-electrode in a 0.7 mL cell at 25°C using an Instech System and DUO18 (World Precision Instruments, Inc.) amplifier appropriately set to reveal slight oxygen concentration changes. For all the experiments the ambient O_2 concentration was considered to be 260 μM.

Generation of two-dimensional maps

Proteins were solubilized in 125 mL of the solubilization buffer (Sol B)(7 M urea, 2 M thiourea, 2.5% IPG buffer pH 3-10, 1% DTT, 0.04 M Tris, 2% Triton X-100, 2% CHAPS, a few grains of bromophenol blue), vigorously shaken at room temperature for at least 16 h and loaded on IPG strip gel by passive reswelling for at least 20 h at 20°C. Before the two dimensional analysis, the proteins focused on the IPG strip were reduced and alkylated through submersion in equilibration buffer (EqB): 0.05 M Tris-Cl pH 8.8, 6 M urea, 30% v/v glycerol, 2% w/v SDS) plus 1% DTT for 15 min with gently mixing, and then in EqB plus 2.5% iodoacetamide for 15 min with gently mixing. After alkylation IPG strips were washed in electrode buffer (192 mM glycine, 25 mM Tris, 0.1% SDS) and left in the air, just before loading on the minigel. A thin layer of 0.5% melted agarose LM (dissolved in electrode buffer, plus bromophenol blue) was added on the top of the gel. The two dimensional study was performed in a Biorad Mini Protean 3 Dodeca Cell chamber (Hercules, CA, USA). The gels were stained with colloidal Coomassie for image analysis and protein identification.[4]

Image analysis and statistical analytic strategy

Spots differently expressed were identified by Progenesis SameSpots software (Non Linear Dynamics, Newcastle-Upon-Tyne, UK).[5] Colloidal Coomassie-stained gels were aligned by both manual and automatic alignment of the images. All spots were pre-filtered and manually checked before applying the statistical criteria. We considered only spots that changed more than 2.5-fold comparing control versus β-thalassemic erythroid precursors. Quantitation and comparison were based on the normalized volume of spots and statistical analysis was based on ANOVA one way test, with *P* values less than 0.05 considered statistically significant. The Progenesis SameSpots software automatically creates the dependence of logarithm of normalized volume on the sample grouping of all spots. Experimental *pI* and molecular weight values were estimated using the protein molecular weight marker, together with values of known and identified proteins.

MALDI-TOF MS analysis and database search

The spots from two-dimensional gels differently expressed in colloidal Coomassie-stained gels were excised, destained (in destaining solution: 50% acetonitrile, 5 mM NH_4HCO_3), dehydrated in 100% acetonitrile and digested overnight at 37°C with 20 μL of trypsin solution (0.01 mg/mL trypsin, 5 mM NH_4CO_3). Mass spectra analysis was performed using a Tofspec SE (Micromass, Manchester, UK) or MALDI-Micro MX mass spectrometer with PSD technology (Micromass, Manchester, UK). Peptide spectra were obtained in the positive ion mode over the m/z range of 800–4000 Da range or 1000-3000 Da in reflectron mode. Peptide solution was prepared mixing equal volumes of matrix (matrix: saturated α-cyano-4-hydroxy cinammic acid solution in 40% acetonitrile, 60% of 0.1% trifluor acetic acid). 100–120 laser shots were summed for each mass spectrum. Database searching was performed using the measured peptide masses against the Swiss-Prot database (*taxa human*) using the MASCOT search engine (Matrix Science Ltd, London, UK). Only protein identifications with significant MASCOT scores ($P<0.05$) were taken into consideration. A mass accuracy of 0.3 Da and a single missed cleavage were allowed for each matching peptide. Searches were not constrained by *pI* or molecular weight.[4,5]

Immunoblot analysis

For one-dimensional electrophoresis, cells were solubilized as described by Karur *et al.*[6] with few changes. Briefly, cells (5×10^6) were lysed by gently vortexing in medium containing bicine 25 mM, NaF 25 mM, NaCl 0.4 M, EDTA 20 mM, Triton 1.5%, Na_3VO_4 1 mM, benzamidine 3 mM and a cocktail of protease inhibitors (Roche, Germany). At 20 min, the cell lysates were sonicated and SDS 0.1%, DTT 100 mM were added; the samples were then vortexed five times over a 5-min interval. The cleared lysates were used for protein concentration studies. We ran twin gels, one stained with colloidal Coomassie to control the protein loading and the other transferred to membrane for immunoblot analysis with specific antibodies. Biliverdin reductase (BVR; Stressgen, Ann Arbor, MI, USA), peroxiredoxin-1 (PRDX1; abcam, Cambridge, UK), peroxiredoxin-2 (PRDX2; LabFrontier, Seoul, Korea), peroxiredoxin-6 (PRDX6; Sigma, St Louis, MO, USA), catalase (ab 1877; Abcam, Cambridge, UK), heat shock protein 27 (HSP27; sc-1048, SantaCruz Biotechnology, CA, USA), heme-oxygenase-1 (HO-1; H-105, sc 10789 SantaCruz Biotechnology, CA, USA), heme-oxygenase-2 (HO-2; H-73, sc 11361 SantaCruz Biotechnology, CA, USA); ALAS-2 (sc32334; SantaCruz Biotechnology, CA; USA). Secondary anti-rabbit, anti-goat and anti-mouse antibodies were from GE healthcare (Little Chalfont, UK). ECL-Plus (Amersham, UK) was used as the revealing system.

Peroxiredoxin-2: cytofluorimetric and immunoprecipitation assays

Erythroid precursors, permeabilized as previously described,[7,8] were probed with specific anti-PRDX2 antibody (abcam, Cambridge, MA, USA) as the primary antibody followed by anti-mouse-Alexa Fluor 488 (Invitrogen, Life Technologies, Carlsbad, CA, USA) as the secondary antibody, were subjected to cytofluorimetric analysis using a FACS-Canto cytometer (BD Biosciences, Milan, Italy). Data were analyzed with FACS DIVA software (BD Biosciences). The extent of the possible autofluorescence from heme breakdown products in the Alexa Fluor 488 channel was determined through fluorimetric measurements in a Jasco FP750 Spectrofluorimeter. The extent of the possible autofluorescence from heme breakdown products in the Alexa Fluor 488 channel was determined through fluorimetric measurements in a Jasco FP750 Spectrofluorimeter by exciting the control and H_2O_2-treated samples (both at 14 days of culture), treated in the same way as for FACS analyses, in a 1 mL-cuvette at 495 nm and evaluating the emission spectra from 500 to 700 nm. When excited at 495 nm, Alexa Fluor 488 emits with a maximum centered at 519 nm. Following excitation at 495 nm, the emission spectra from 500 to 700 nm of control and H_2O_2-treated cells in the absence or presence of Alexa Fluor 488 were determined and showed that autofluorescence accounts for less than 1% of the signal recorded at 519 nm (*Online Supplementary Figure S2A*).

Whenever indicated, PRDX2 was immunoprecipitated from cell lysates (11×10^6 cells). Lysates were pre-cleared by incubation for 1 h at 4 °C in a rotating wheel with 30 μL of protein A immobilized to Trysacryl (Pierce Chemical Co., Rockford, IL, USA). After pre-clearing, lysates were incubated with 20 μL of protein A-Trysacryl pre-adsorbed with specific anti-PRDX2 antibody (LabFrontier, Korea) followed by washing in a medium containing bicine 50 mM, pH 7.4, sodium vanadate 0.2 mM, NaF 1mM, EDTA 1mM and protease cocktail inhibitor. The amount of heme bound to PRDX2 was determined as reported below on the immunoprecipitated PRDX2, which was then analyzed by immunoblot with specific anti-PRDX2 antibody (abcam, Cambridge, MA, USA). The amount of immunoprecipitated PRDX2 was evaluated by densitometric analysis.

Measurements of heme levels

The heme concentration in the control and β-thalassemic cells was determined by a spectrophotometric method that exploits heme peroxidative activity.[9] Hydrogen peroxide oxidizes a dye, chlorpromazine, and heme, acting as a peroxidase, increases the rate of the reaction. Eight hundred microliters of an acetic acid/phosphoric acid solution, 5-10 μL of a control or β-thalassemic sample, and 250 μL of the chlorpromazine solu-

tion (100 mg in 10 mL of water) are mixed in a 10 mm path length cuvette. After collecting the blank, 500 μL of a hydrogen peroxide solution 3% (v:v) are also added to give a final concentration of 0.38 M. After mixing, the absorbance change at 528 nm is monitored for about 5 min with a Beckman DU 650 Spectrophotometer coupled with a thermostatic cell at 25 °C. In order to assess heme concentrations, a calibration curve was obtained, using increasing concentrations of hemin chloride standards, prepared in water (from 0.02 to 1 μM) and determined spectrophotometrically considering that hemin chloride has a ε_M= 58.4 mM^{-1} cm^{-1} at 385 nm in 100 mM NaOH. The heme concentrations obtained were normalized for cell numbers.

Quantitative real-time polymerase chain reaction

For quantitative real time (qRT) polymerase chain reaction (PCR), total RNA was isolated from cell pellets on days 7 and 14 of culture by the method of Chomczynski and Sacchi.[10] cDNA was synthesized using 2 μg RNA as the template in a 20 μL reaction with Superscript III reverse transcriptase (Invitrogen, Life Technologies, Carlsbad, CA, USA). qRT-PCR was performed with SYBR Green PCR Master Mix (Applied Biosystems) using the Applied Biosystems Model 7900HT Sequence Detection System. Further details of the methods and primer sequences are available on request and in *Online Supplementary Table S1*. All PCR reactions were performed in triplicate. Relative gene expression was calculated using the $2^{-\Delta Ct}$ method, in which Ct indicates cycle threshold, the fractional cycle number at which the fluorescent signal reaches the detection threshold. The ΔCt was computed by calculating the difference of the average Ct between the gene of interest and the internal control *GAPDH*. The data are presented as mean ± the standard deviation (SD).

The analysis of expression of α-, β- and γ-globin genes was performed using the TaqMan primers (HBB Hs00747223_g1; HBA Hs00361191_g1; HBG Hs00361131_g1 Applied Biosystem).

Measurements of erythroid δ-aminolevulinate-synthase activity

Recombinant δ-aminolevulinate-synthase (ALAS-2) was a generous gift from Dr. Gloria C. Ferreira (University of Tampa, FL; USA). ALAS-2 activity was determined as reported by Hunter *et al.*,[11] using a spectrophotometric continuous assay and monitoring NADH increase (equal to 5-aminolevulinate formation) at 340 nm with a Jasco V-650 double beam spectrophotometer coupled to a thermostatic cell set at 30°C. The concentration of ALAS-2 was 5-10 μg/mL. In erythroid precursors, ALAS-2 activity was determined in cytoplasmic fractions (20 μL) from controls, controls treated with H_2O_2 or β-thalassemic cells at days 7 and 14 of culture. These samples were diluted into the same reaction mixture as above in the presence of 10 μM pyridoxal 5'-phosphate and the ALAS-2 activity was assayed as described above. A control experiment of recombinant ALAS-2 activity in the presence of the solubilization medium (bicine 25 mM, NaF 25 mM, NaCl 0.4 M, EDTA 20 mM, Triton 1.5%, pH 7.4) assessed that under these experimental conditions the recovery of ALAS-2 activity was 100%. Tertiary and secondary structure analyses were performed by circular dichroism measurements of ALAS-2 alone or in the presence of saturating hemin or hydrogen peroxide concentrations and carried out in the near (260-550 nm) and far (190-240 nm) UV region with a 1 or 0.1 cm path length cuvette, respectively, in a Jasco-J110 spectropolarimeter by averaging three spectra.

Cloning, expression and purification of human peroxiredoxin-2

Human cDNA of *PRDX2* was purchased from OriGene. By means of PCR amplification, using appropriate oligonucleotide primers, the *PRDX2* gene was amplified and subcloned into the bacterial expression vector pET22b+. The pET22b+/*PRDX2* construct was sequenced to verify the correct cloning and was expressed in *E. coli* BL21 following induction with 0.4 mM IPTG and purification through affinity purification by IMAC (using a HiPrep GE Healthcare column). The protein has a C-terminal His-tag (6 His residues) for efficient purification. For purification the bacterial extract was loaded onto an affinity column equilibrated with 50 mM Tris-HCl, pH 7.4 containing 0.5 M NaCl and 20 mM imidazole. PRDX2 was eluted by applying a linear gradient with the same buffer as above except that the concentration of imidazole was 500 mM. The protein elutes at 200 mM imidazole and was immediately subjected to filtration with an Amicon-Ultra-3k device to concentrate and exchanged with sodium phosphate buffer pH 7.5 containing 0.1 mM DTT. The His-tag was then removed by thrombin (Sigma) cleavage. Briefly, His-tagged PRDX2 was incubated with thrombin (1 mg:10 units) at 20°C for 15 h and then separated by gel filtration analysis on a Sephacryl (16/60) in a Akta Prime FPLC system. The protein appears as a single band on SDS-PAGE and was stable at -20°C for 3 months.

Silencing of peroxiredoxin in K562 cells and exposure to oxidative stress

We transfected 0.8×10^6 K562 cells with 2.5 μg Expression Arrest pSM2 retroviral shRNAmir plasmids (empty vector and non-silencing vector as controls, and shRNAmir plasmid against *PRDX2*) (Open Biosystems) using TransIT-LT1 transfection reagent (Mirus). To select stable pools, 48 h after transfection, the cells were cultured with 2 μg/mL puromycin (Invitrogen). The sequence of shRNA is reported in *Online Supplementary Table S2*. To induce K562 erythroid differentiation 50 μM hemin (Sigma, St. Louis, MO, USA) was added to the culture medium of the empty vector, non-silencing vector and shRNAmir plasmid against *PRDX2*. Samples were collected at specific time points (after transfection): before hemin addition, and at days 4 and 6 after hemin addition. Erythroid differentiation was assessed by FACS analysis for glycophorin A (CD235A). The total samples evaluated in the present study were obtained from three independent experiments, each performed in duplicate, as described above for each transfection method. In order to evaluate the effect of oxidative stress on shK562, confluent cells suspended in phosphate-buffered saline (PBS) at a concentration of 1.5×10^5 cells/mL were incubated for 30 min with 50 μM H_2O_2 as previously reported.[12] Stock solutions of freshly diluted H_2O_2 in PBS were used for all cell treatment experiments. H_2O_2 was removed by centrifugation and repeated washing. Cell viability was assayed after 24 and 48 h following treatment with H_2O_2. H_2O_2 was removed and cells

resuspended in Iscove's complete medium (1.5×10^5 cells/mL). Cell viability was assessed by the trypan blue dye exclusion test.[13] Values were expressed as a ratio of trypan blue-positive cells at the times indicated (24 and 48 h).

Kinetic and inhibition assays of peroxidase activity of peroxiredoxin-2

The kinetic parameters of the peroxidase reaction catalyzed by recombinant PRDX2 were measured by incubating 2-5 μg/mL of PRDX2 with various concentrations of H_2O_2 (0.2-500 μM) in the presence of the coupled assay system consisting of 3 μM thioredoxin, 1.5 μM thioredoxin reductase and 300 μM NADPH in 50 mM Hepes, pH 7.5 at 30°C. The rate of decrease at 340 nm due to NADPH oxidation gives the initial velocity at each H_2O_2 concentration. The data obtained were fitted to a Michaelis-Menten equation with a modification that accounts for substrate inhibition:

$$v_0 = k_{cat} /(1 + (K_m /[H_2O_2]) + ([H_2O_2]/K_i) \quad (1)$$

The inhibition assays of PRDX2 in the presence of hemin were carried out as follows. To a solution containing 2-5 μg/mL PRDX2, different concentrations of hemin (10-500 nM) were added and the remaining activity was assayed with the coupled thioredoxin-thioredoxin reductase-NADPH system in the presence of 8 μM H_2O_2. Initial rate values were plotted as a function of log hemin concentrations and an IC_{50} value was obtained by fitting the data to a dose-response sigmoid curve.

Data analyses were performed using non-linear curve software Origin 7 (Origin Laboratory Corporation, Northampton, MA, USA).

Equilibrium binding studies of recombinant peroxiredoxin-2 with hemin

PRDX2 (4.5 μM) intrinsic fluorescence spectra were measured by exciting the enzyme at 295 nm, at which tryptophan residues have their absorbance maximum, and recording spectra in the interval from 300 to 600 nm with a Jasco FP750 spectrofluorimeter in 50 mM sodium phosphate buffer, pH 7.4, at 25°C. Increasing concentrations of hemin (10-500 nM) were then added and the change in tryptophan quenching at 338 nm was plotted versus hemin concentration. Given that binding occurs at a much lower concentration of hemin than of Prdx2, data points were fitted using the tight binding hypothesis with a quadratic equation to obtain the K_d value.

$$Y = Y_{max}\left\{[E]_t + [L] + K_d - \sqrt{([E]_t + [L] + K_d)^2 - 4[E]_t[L]}\ \right\}/2[E]_t \quad (2)$$

where Y represents the measured quenching extent in percentage, Y_{max} the 100% quenching (that is the variation of the value of emission fluorescence at 338 nm exhibited by the enzyme alone minus that in the presence of saturating ligand concentration, [L] represents the hemin concentration, $[E]_t$ the total enzyme concentration and K_d, the equilibrium dissociation constant value.

Absorbance spectra of 3 μM PRDX2 in the presence of 3 μM hemin were measured in 50 mM Hepes, pH 7.5 in the absence or presence of a 1000-fold molar excess of sodium dithionite or 1 mM DTT in a Jasco V-650 Spectrophotometer at 25°C.

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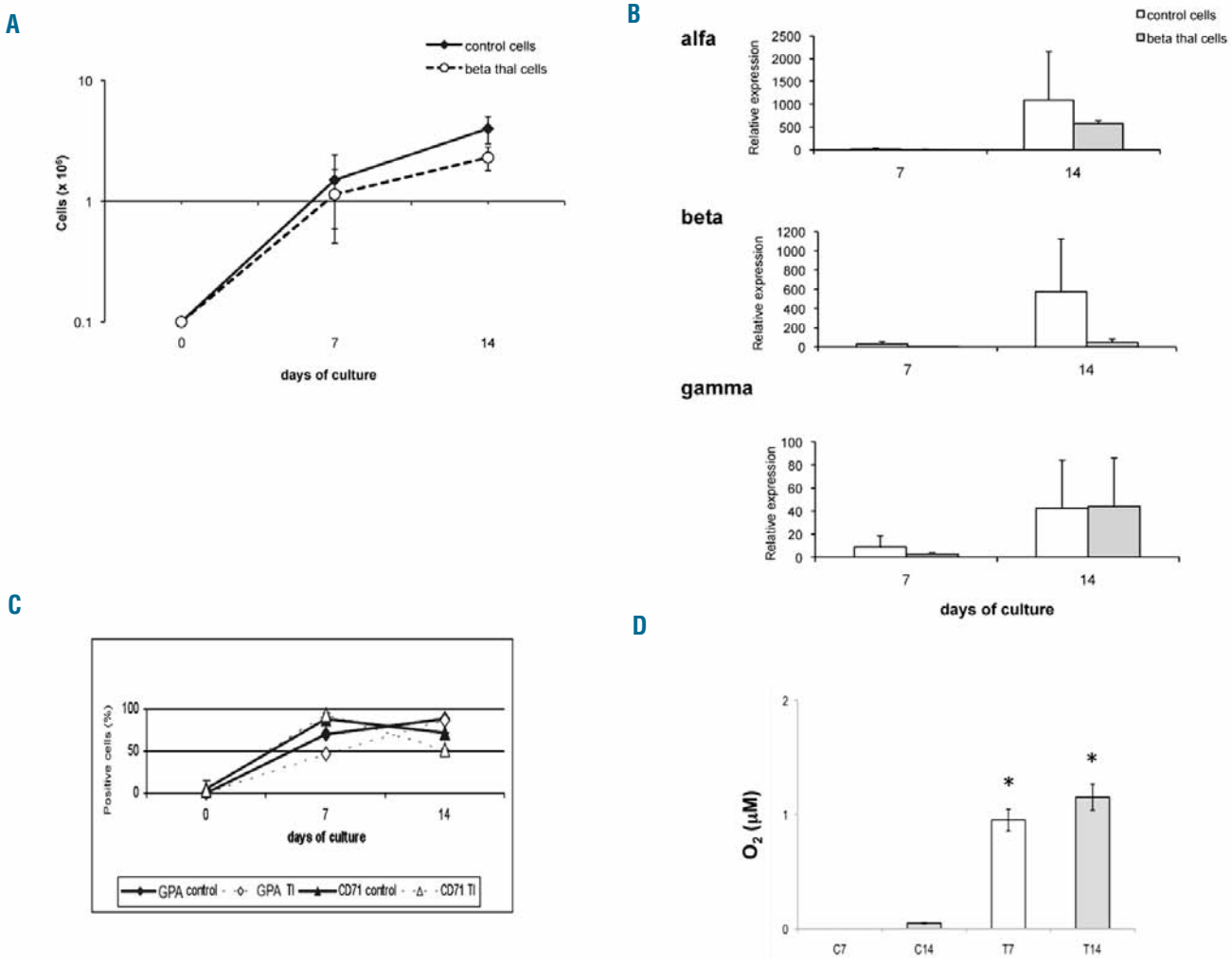
Online Supplementary Table S1. List of primers for genes analyzed by quantitative RT-PCR.

Gene	Forward Primer Sequence (5'→ 3')	Reverse Primer Sequence (5'→ 3')
BVR	ACACATAGGAGACCAGCCACT	TGTTTGGAGATGACCCTTGAGG
PRDX2	ACAAAGGGAAGTACGTGGTCCTC	GCTGAACGCGATGATCTCG
HSP27	AGACCAAGGATGGCGTGG	GGAGATGTAGCCATGCTCGTC
CATALASE	CCAAATACTCCAAGGCAAAGGT	CTCCAGCAACAGTGGAGAACC
HO-1	TGACCCGAGACGGCTTCA	TGTTGCGCTCAATCTCCTCC
HO-2	AAGACCAAAGAGAGGATCGTGG	GGCCTGGTCCAGTTCATTGA
ALAS-2	GCCACACAGGAGACCCTGCA	GAAACTTACTGGTGCCTGAG
GAPDH	CCACATCGCTCAGACACCAT	AGTTAAAAGCAGCCCTGGTGAC

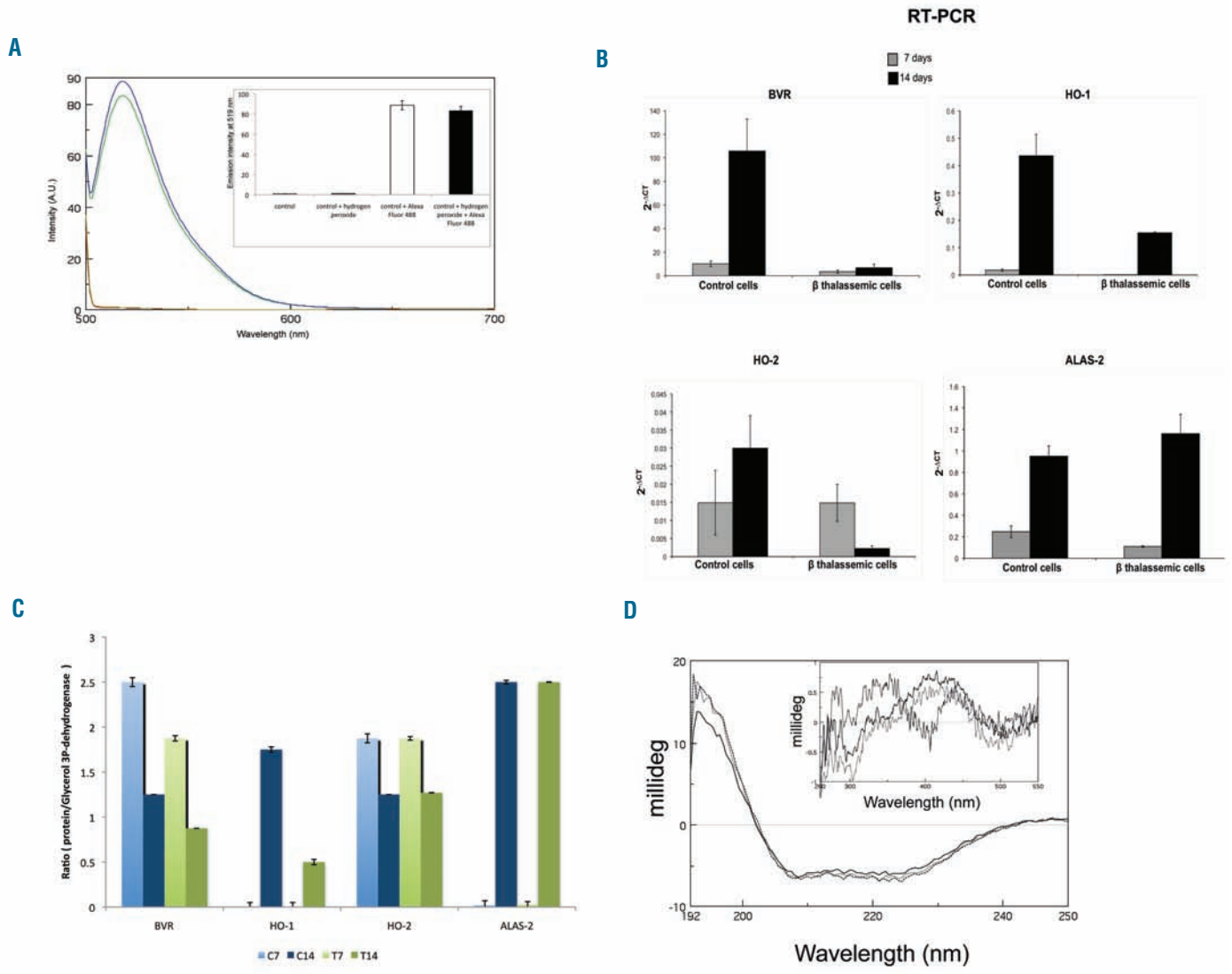
Online Supplementary Table S2. List of shRNA used in trasfection experiments.

Sequence of non-silencing shRNAmir:
TGCTGTTGACAGTGAGCGATCTCGCTTGGGCGAGAGTAAGTAGTGAAGCCACAGATGTACTTACTCTCGCCCAAGCGAGAGTGCCTACTGCCTCGGA.

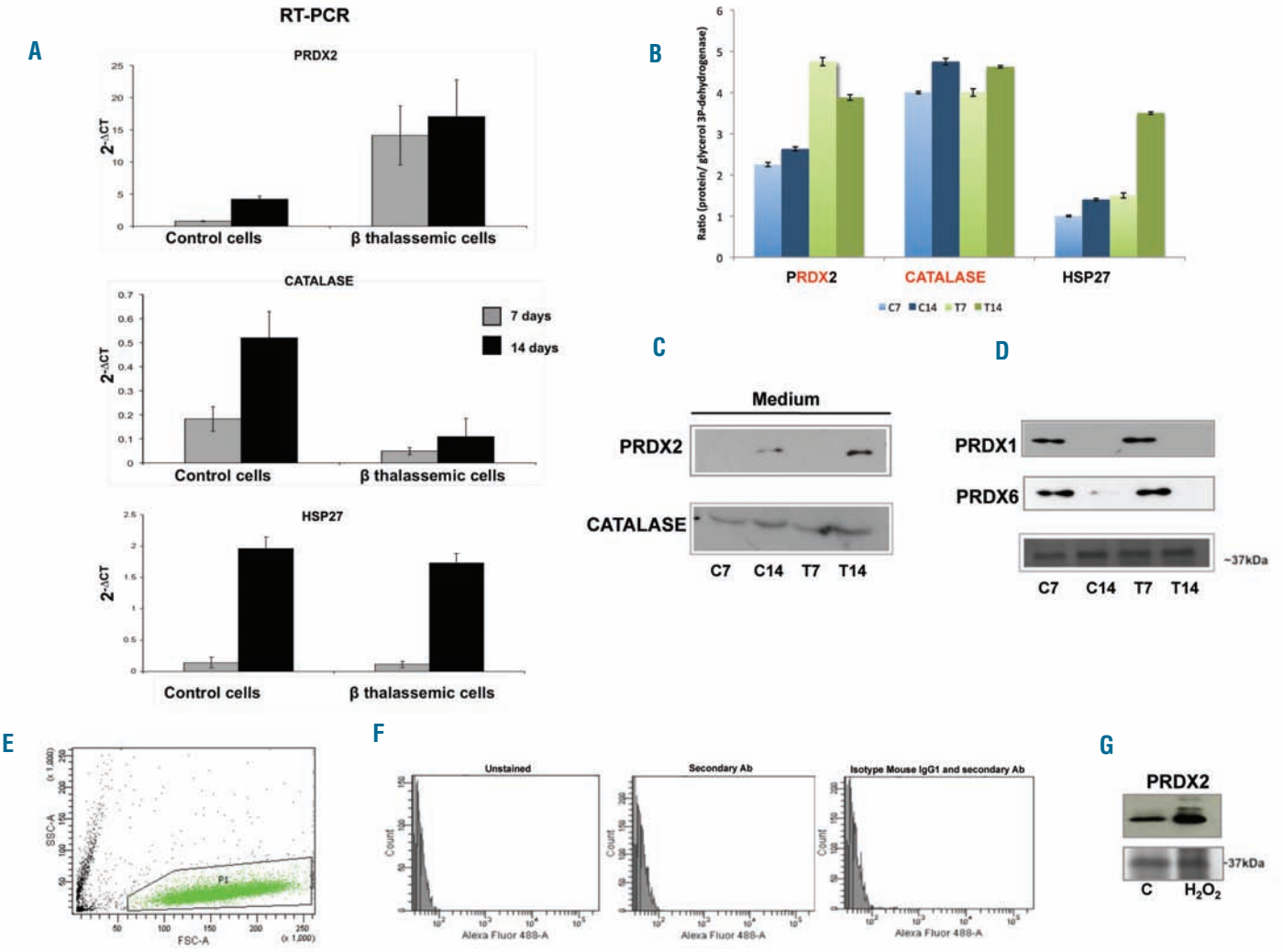
Sequence of silencing *PRDX2* shRNAmir:
TGCTGTTGACAGTGAGCGAGCCAGATCACTGTTAATGATTTAGTGAAGCCACAGATGTAAATCATTAACAGTGATCTGGCGTGCCTACTGCCTCGGA



Online Supplementary Figure S1. (A) Cell proliferation of erythroid precursors derived by *in vitro* liquid culture of CD34$^+$ cells isolated from peripheral blood of normal (control cells) and β-thalassemic (beta thal cells) subjects (n=9). (B) Molecular analysis for erythroid expression of the following genes: α-, β-, γ-globins in normal (control) and β thalassemic (T) erythroid precursors. Gene expression was normalized to the internal control *GAPDH* for each data point, the data are presented as mean±SD of five experiments. (C) Surface marker expression in controls and and β thalassemic erythroid cells. The cells were harvested on days 0, 7, and 14, washed and stained with fluorescent antibody against CD71 and glycophorin A (GPA). The data are presented as the average ±SD of six experiments. (D) Reactive oxygen species (ROS) production in normal (C) and β-thalassemic (T) erythroid precursors at days 7 and 14 of culture measured as dioxygen formation following addition of catalase or superoxide dismutase to the reaction mixtures (see *Design and Methods* section for details). Data are presented as means ± standard deviations (n=5); * $P<0.05$ compared to control cells.



Online Supplementary Figure S2. (A) Emission spectra of control or H_2O_2-treated erythroid precursors in the presence or absence of Alexa Fluor 488. Samples were treated as for FACS analyses and subjected to excitation at 495 nm in a Jasco FP750 spectrophotometer. Colors are as follows: yellow and brown: control and H_2O_2 treated cells in the absence of Alexa Fluor 488, respectively; green and blue: control and H_2O_2 treated cells in the presence of Alexa Fluor 488, respectively. The small variability in the maximum intensity value is within the experimental 10% error incidental to fluorimetric analyses. Spectra were measured three times, preparing the samples appropriately each time. One representative experiment is shown. Inset: extent of 519 nm emission band in the different cell types and conditions, white bar: control erythroid precursors with and without hydrogen peroxide, black bar: control erythroid precursors with and without hydrogen peroxide in the presence of Alexa Fluor 488. (B) RT-PCR expression of biliverdin reductase (*BVR*), heme oxygenase-1 (*HO-1*), heme-oxygenase-2 (*HO-2*) and 5-δ aminolevulinate synthase (*ALAS-2*) in control (on the left) and β thalassemic (on the right) cells at day 7 (gray bars) and 14 (black bars) of culture. Experiments were performed in triplicate. Error bars represent the standard deviations (mean ± SD; $P<0.01$ by two-tailed t test). (C) Protein expression for biliverdin reductase (BVR), heme-oxygenase -1 (HO-1), heme-oxygenase 2 (HO-2) and ALAS-2 was assessed by immunoblot analysis and adjusted for the expression of the reference band identified by mass spectrometry as glycerol 3P-dehydrogenase cytoplasmic domain (accession number: P21695, 14 % sequence coverage). Data from densitometric analysis are presented as means ± SD, n= 9. (D) CD spectra of ALAS-2 in the presence or absence of hemin or hydrogen peroxide. CD spectra in the far UV region of ALAS-2 (-), ALAS-2 in the presence of 5 mM hemin (- - -) and ALAS-2 in the presence of 5 mM hydrogen peroxide (. . .). The inset shows the near-UV and visible region. Symbols are the same as above. Measurements were carried out in 20 mM Hepes, pH 7.2.



Online Supplementary Figure S3. (A) RT-PCR expression of peroxiredoxin-2 (*PRDX2*), *CATALASE* and heat shock protein 27 (*HSP27*) genes in control (on the left) and β-thalassemic (on the right) cells at day 7 (gray bars) and day 14 (black bars) of culture. Experiments were performed in triplicate. Error bars represent the standard deviations (mean ± SD; $P<0.01$ by two-tailed t test). (B) Protein expression for peroxiredoxin-2 (PRDX2), catalase and heat shock protein 27 (HSP7) was assessed by immunoblot analysis and adjusted for the expression of the reference band identified by mass spectrometry as glycerol 3P-dehydrogenase cytoplasmic domain (accession number: P21695, 10 % sequence coverage). Data from the densitometric analysis are presented as means ± SD, n= 9. (C) Peroxiredoxin-2 (PRDX2) and catalase protein levels in the media of normal (C7, C14) and β-thalassemic (T7 and T14) erythroid cultures. 80 μg of proteins from purified media were loaded (see also *Design and Methods* section). The gel is representative of nine other showing similar results. (D) Immunoblot analysis of peroxiredoxin-1 (PRDX1) and peroxiredoxin-6 (PRDX6) protein expression in erythroid cells from normal (C7, C14) and β-thalassemic (T7 and T14) erythropoiesis. We ran twin gels one stained with colloidal Coomassie and the other transferred to membrane for immunoblot analysis with specific antibodies. We showed on the colloidal Coomassie stained gel the band at ~ 37 kDa used as the loading control and identified by mass spectrometry as glycerol 3P-dehydrogenase cytoplasmic domain (accession number: P21695, 12 % sequence coverage). One representative gel from the ten other with similar results is presented. (E) Strategy of flow cytometric analysis of erythroid precursors referred to Figure 4B. Correlative display of orthogonal light scatter (SSC: Y-axis) *versus* forward light-scatter (FSC: X-axis). of erythroid progenitors (region P1). (F) Controls for flow cytometric analysis referred to Figure 4B, as follow: unstained cells, cells stained with anti-mouse secondary antibody (Ab), cells stained with mouse IgG1 (isotypic control for anti-peroxiredoxin-2 antibody) and secondary antibody; (G) Immunoblot analysis of peroxiredoxin-2 (PRDX2) protein expression in normal erythroid cells exposed to H_2O_2 (16 mM) at 14 days of culture referred to Figure 4B. We ran twin gels: one stained with colloidal Coomassie and the other transferred to membrane for immunoblot analysis with specific antibodies. We showed on the colloidal Coomassie stained gel the band at ~ 37 kDa used as the loading control and identified by mass spectrometry as glycerol 3P-dehydrogenase cytoplasmic domain (accession number: P21695, 14 % sequence coverage). One representative gel from the three others with similar results is presented.