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The iron chelator deferasirox affects redox signalling in haematopoietic stem/progenitor cells

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Summary

The iron chelator deferasirox (DFX) prevents complications related to transfusional iron overload in several haematological disorders characterized by marrow failure. It is also able to induce haematological responses in a percentage of treated patients, particularly in those affected by myelodysplastic syndromes. The underlying mechanisms responsible for this feature, however, are still poorly understood. In this study, we investigated the effect of DFX-treatment in human haematopoietic/progenitor stem cells, focussing on its impact on the redox balance, which proved to control the interplay between stemness maintenance, self-renewal and differentiation priming. Here we show, for the first time, that DFX treatment induces a significant diphenyleneiodonium-sensitive reactive oxygen species (ROS) production that leads to the activation of POU5F1 (*OCT4*), *SOX2* and *SOX17* gene expression, relevant in reprogramming processes, and the reduction of the haematopoietic regulatory proteins CTNNB1 (β-Catenin) and BMI1. These DFX-mediated events were accompanied by decreased CD34 expression, increased mitochondrial mass and up-regulation of the erythropoietic marker CD71 (TFRC) and were compound-specific, dissimilar to deferoxamine. Our findings would suggest a novel mechanism by which DFX, probably independently on its iron-chelating property but through ROS signalling activation, may influence key factors involved in self-renewal/differentiation of haematopoietic stem cells.

Keywords: deferasirox, reactive oxygen species, haematopoietic stem cell, differentiation, myelodysplastic syndromes.

Haematopoietic homeostasis and regeneration is dependent on the self-renewal capacity of haematopoietic stem cells (HSCs), as well as their ability to differentiate into multiple haematological lineages. The balance between HSC self-renewal and commitment is controlled by a combination of cell-intrinsic and external regulatory cues (Suda *et al*, 2005). Among these, oxygen tension is thought to play a significant role in HSC maintenance, survival and metabolism (Parmar *et al*, 2007). In the adult, HSCs reside in the bone marrow within a specialized, poorly perfused tissue microenvironment whereas after mobilization, they reach a normoxic milieu in a perivascular niche (Naka *et al*, 2008; Warren & Rossi, 2009). The oxygen content that surrounds the cells influences the generation of reactive oxygen species (ROS) (Hao *et al*, 2011). Previous studies showed that mobilized human $CD34^+$-haematopoietic stem/progenitor cells (HSPCs) constitutively generate low levels of ROS attributable to both the mitochondrial respiratory chain and NADPH oxidases (Piccoli *et al*, 2007a). Under physiological circumstances, low and moderate levels of ROS are not detrimental for cells, but rather they act as signal mediators regulating the activation of growth/proliferation and differentiation processes (Sauer *et al*, 2001; Boonstra & Post, 2004; Noble *et al*, 2005; Piccoli *et al*, 2005).

Disturbance of HSC differentiation characterizes the myelodysplastic syndromes (MDS) (Vardiman *et al*, 2009).

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MDS patients can develop severe anaemia and require blood transfusions. The resulting iron overload is a common complication of MDS management and is treated with iron chelators (Gattermann *et al*, 2010). Intriguingly, several retrospective (Molteni *et al*, 2013; Molica *et al*, 2014) and prospective (Gattermann, 2012; Gattermann *et al*, 2012; List *et al*, 2012; Angelucci *et al*, 2014) clinical reports have shown an additional positive effect of the iron chelator deferasirox (DFX) on haematopoiesis (reviewed in Guariglia *et al*, 2011), leading to a partial reduction or a complete interruption of transfusions in a limited but significant proportion (10–15%) of MDS patients. Of note, none of the reported studies focused on the haematological response as end-point.

Deferasirox is a low molecular weight and high lipophilic drug allowing it to be taken orally, unlike deferoxamine (DFO), which has to be administered intravenously. Notably, other commonly used iron chelators, such as DFO or deferiprone, seem to exhibit a limited, if any, similar beneficial side effect (Jensen *et al*, 1996; Pootrakul *et al*, 2004). It is conceivable that this is due to some specific ability of DFX to restore correct HSC differentiation, although the underlying molecular mechanism remains to be defined.

In this study we sought to investigate the direct effect of DFX on isolated normal human HSPCs, focussing our attention on possible molecular targets whose expression is orchestrated by ROS signalling.

Methods and materials

Cell samples

Ethical approval was obtained from the local ethical committees for sampling from normal donors. Peripheral blood mononuclear cells (PBMCs) from healthy donors following informed consent, were isolated by density separation over a Ficoll-Paque™ (GE Healthcare, Stockholm, Sweden) gradient (460× g for 30 min). PBMCs were washed three times with phosphate-buffered saline (PBS) pH 7·4/1 mmol/l EDTA (Life Technologies, Paisley, UK). Cells were counted and their viability assessed by trypan blue exclusion. Peripheral HSPCs were obtained from healthy donors, following informed consent, by apheresis performed for transplant purpose, after mobilization following recombinant granulocyte colony-stimulating factor treatment. HSPCs were collected via a Code/Spectra-device and positively selected by the super-paramagnetic iron-dextran particles directly conjugated to anti-CD34 monoclonal antibodies, as previously described (Tabilio *et al*, 1997). After thawing, cell viability, determined by trypan blue exclusion, was typically between 85% and 95%. PBMCs and HSPCs were maintained in RPMI medium supplemented with 10% fetal bovine serum, 100 u/ml penicillin, 100 μg/ml streptomycin, and 2 mmol/l L-glutamine in a humidified atmosphere with 5% CO_2 at 37°C for the indicated time periods. DFX (Novartis, Basel, Switzerland), DFO (Sigma Aldrich, St. Louis, MO, USA) or diphenyleneiodonium (DPI) (Sigma Aldrich) were added as indicated.

Cell viability

After treatment, the number of viable/death cells was counted by trypan blue dye exclusion using the Burker counting chamber. The result was expressed as a percentage relative to untreated control.

Assessment of intracellular ROS

The intracellular ROS of PBMCs and HSPCs was measured using flow cytometric analysis. An aliquot (2×10^5) of cells per condition was incubated for 15 min at 37°C with 4 μmol/l of 2′,7′-dichlorodihhyrofluorescein-diacetate (H_2DCF-DA) (Sigma Aldrich). After washing in PBS, 10^4 events for each sample were acquired and analysed using a FACS Calibur flow cytometer with CellQuest software (Becton Dickinson Bioscience – BDB, San Jose, CA, USA).

RNA extraction, reverse transcription and real-time-polymerase chain reaction analysis

Total cellular RNA was isolated by Trizol reagent (Life Technologies, Paisley, UK), quantified by NanoDrop® ND-1000 Spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA) and its quality assessed by electrophoresis on agarose gel (1%). Total RNA (1 μg) was used in a reverse transcription reaction using the Transcriptor first strand cDNA synthesis kit (Roche Diagnostics, Penzberg, Germany) according to the manufacturer's instructions. Quantitative real-time polymerase chain reaction (PCR) assays were performed in duplicate using the QuantiTect Primer Assay (Qiagen, Basel, Switzerland) or primer designed using the Primer3 software (http://bioinfo.ut.ee/primer3-0.4.0/) (Table I). For each reaction, 2 μl of the complementary DNA were added to 16 μl of Light Cycler® 480SYBR Green I Master) (Roche Diagnostics) and primer pair, in a total volume of 20 μl. Quantification of the mRNA levels was performed on a

Table I. Features of the primers used for real-time polymerase chain reaction.

Transcript	T_{ann} (°C)	Length (bp)	NCBI Ref Seq or sequence
POU5F1	60	77	NM_203289
SOX2	55	64	NM_003106
NANOG	55	202	Forward: aagacctggttccagaacc
			Reverse: tccaggttgaattgttcc
MYC	55	129	NM_002467
SOX17	60	119	NM_022454
GAPDH	60/55	135	Forward: aggctgagaacgggaagc
			Reverse: ccatggtggtgaagacgc

NCBI Ref Seq: National Center for Biotechnology Information Reference Sequence (http://www.ncbi.nlm.nih.gov/RefSeq/) identifier.

LightCycler® 480 real-time PCR Instrument. The following protocol was used for the PCR reactions, initalization at 95°C for 3 min followed by 40 cycles of 95°C for 10 s, 55°C or 60°C for 10 s, 72°C for 10 s. The melting program was 95°C for 5 s, 65°C for 1 min 97°C for 10 s. The rate of temperature increase was 1°C/s (or 0·5°C/s), and fluorescence was continuously acquired. The relative amounts of target genes were normalized to *GAPDH* expression by Light Cycler® 480 Software version 1.5 (Roche Diagnostics) using the $2^{\Delta\Delta Ct}$ method.

Western blotting

Aliquots containing 40 μg of protein from each lysate cells were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis on a 10% gel under reducing conditions and then electrotransferred onto a polyvinylidene difluoride membrane (Bio-Rad Laboratories, Hercules, CA, USA) using the Trans Blot Turbo Transfer System. Membranes were probed with primary antibodies [CTNNB1 (β-Catenin); (mouse antiCTNNB1 antibody, 1:1000; BDB), BMI1 (rabbit anti-BMI1 1:500; Santa Cruz Biotechnologies, Santa Cruz, CA, USA)] and then incubated with secondary antibody (horseradish peroxidise-conjugated goat anti-mouse or anti-rabbit respectively, 1:2500; Cell Signaling, Beverly, MA, USA). The same membranes were then stripped and incubated with Actin (mouse anti-Actin antibody 1:5000; Sigma Aldrich). Immune complexes were detected by the ECL chemiluminescence system (Bio-Rad Laboratories), as recommended by the manufacturer. Densitometric analysis was performed using ImageJ 1.45 Software (http//rsb.info.nih.gov/ij/). The intensity of bands, corresponding to CTNNB1 (β-Catenin) or BMI1 proteins, was normalized to the ACTB (β-actin) signal.

Cytofluorimetric analysis of mitochondrial mass and cell surface markers

To label mitochondria, HSPCs were exposed to MitoTracker Green (Molecular Probes, Invitrogen Corp., Carlsbad, CA, USA). Approximately 2×10^5 cells per condition were incubated with 100 nmol/l MitoTracker for 15 min at 37°C, washed with PBS and resuspended in 300 μl of PBS. To detect surface markers, HSPCs were incubated in the dark at room temperature for 15 min with CD71-fluorescein isothiocyanate (FITC), CD19-FITC, CD33-phycoerythrin (PE), CD117-PE, CD45-peridinin chlorophyll (PerCP), CD34-allophycocyanin (APC) directly conjugated monoclonal antibodies (BDB). Cytofluorometric analysis was performed using a FACSCalibur flow cytometer with CellQuest software (BDB). For CD34 and MitoTracker green co-staining, cellular suspensions were incubated with APC-conjugated CD34 antibody at room temperature for 15 min followed by MitoTracker green incubation for 15 min at 37°C. After washing, a total of 10^4 events for each sample were acquired and analysed by FACS Calibur flow cytometer with CellQuest software (BDB).

Statistical analysis

Experimental data are reported as mean ± standard deviation or mean ± standard error of the mean. Data were compared by an unpaired Student's-*t*-test. Differences were considered statistically significant when the *P* value was less than 0·05. All analyses were performed using Graph Pad Prism (Graph Pad Software, San Diego, CA, USA).

Results

DFX, but not DFO, induces ROS production in PBMCs and HSPCs without affecting cell viability

A comparative analysis of the iron chelators DFX and DFO was first carried out on more available samples of PBMCs isolated from healthy donors. Incubation of PBMCs with grading concentrations of either DFO or DFX up to 200 μmol/l for 24 h, did not influence cell viability as assessed by trypan blue exclusion assay (right axis Fig 1A). Next, we tested the impact of DFO and DFX on the intracellular redox balance using H_2DCF-DA, a popular fluorescent probe of intracellular peroxides. Cytofluorimetric analysis of DCF-loaded PBMCs revealed that DFX induced ROS overproduction in a dose-dependent manner with a significant increase at 100 and 200 μmol/l as compared to untreated cells ($P = 0{\cdot}04$ and $P = 0{\cdot}01$, respectively) (left axis Fig 1A). Conversely, DFO treatment did not show any effect. From here on the reported measurements were carried out at compound concentrations of 100 μmol/l. Of note, the chosen concentration of DFX is within the range of its measurable plasma levels in standard 20–30 mg drug/kg/d-treated patients (Galanello *et al*, 2003; Nisbet-Brown *et al*, 2003; Piga *et al*, 2006). The pro-oxidative effect of DFX was also detectable at shorter incubation times, such as 4 h (Fig 1B).

To confirm the specificity of the detected DCF-related signal, DFX-treated PBMCs were co-incubated with DPI, a powerful inhibitor of the main flavoenzymes known to generate ROS, such as the respiratory chain complexes and the NADPH oxidases. As shown in Fig 1C, 20 μmol/l DPI drastically reduced both the basal and the DFX-mediated ROS production to comparable levels.

Next we tested DFX and DFO on isolated blood-circulating $CD34^+$ HSPCs. As with PBMCs, treatment of HSPCs with either of these two iron chelators, although not affecting cell viability, resulted in a specific and DPI-sensitive enhancement of the intracellular ROS generation only by DFX (Fig 1D,E).

DFX affects the expression of stemness transcription factors in HSPCs

To assess the impact of DFX treatment on the HSPCs we decided to evaluate the gene expression of well-established stem cell transcription factors, such as POU5F1 (*OCT4*),

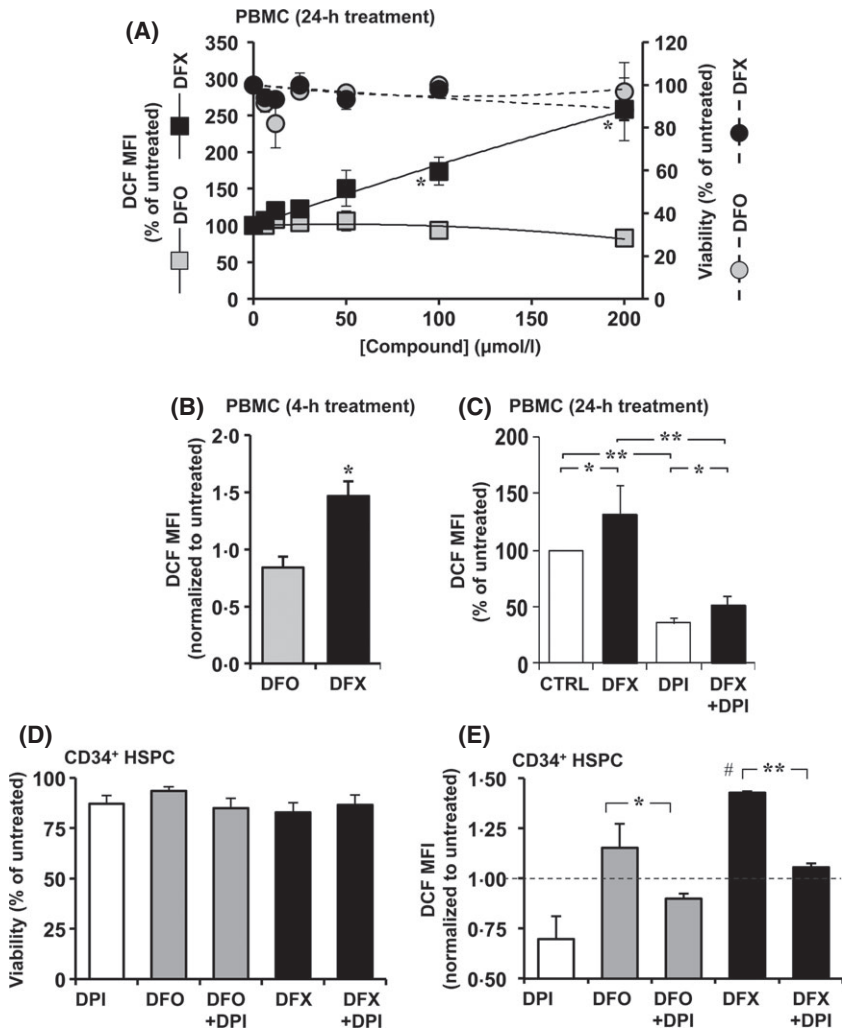

Fig 1. Effect of DFO and DFX on viability and ROS production in PBMCs and CD34$^+$-HSPCs. (A) PBMC viability (right axis, dotted line) and ROS production (left axis, continuous line) after 24-h treatment with DFO (grey symbols) or DFX (black symbols) at the indicated concentrations. ROS production was assessed by flow-cytometry as described in Methods and reported as DC-MFI normalized to untreated cells; *$P < 0{\cdot}05$ and **$P < 0{\cdot}01$ *versus* untreated cells. (B) ROS production assessed as in (A) but following 4-h treatment with 100 μmol/l of either DFO or DFX; *$P < 0{\cdot}05$ *versus* untreated. (C) Effect of 20 μmol/l DPI on ROS production in 24-h treated cells with 100 μmol/l DFX; *$P < 0{\cdot}05$; **$P < 0{\cdot}01$. B, C and D panels represent mean values ± SEM of at least four sets of experiments performed with different preparations of PBMCs. (D) Cell viability and (E) ROS production of isolated HSPCs, assessed as in (A). Isolated HSPCs were incubated for 24 h with 100 μmol/l DFO or DFX in the absence or presence of 20 μmol/l DPI. The indicated values in (D) and (E) are normalized to untreated cells and are means ± standard error of the mean of at least four sets of experiments under each condition performed with different preparations of HSPCs isolated from different healthy donors. *$P < 0{\cdot}05$ and **$P < 0{\cdot}01$ *versus* DPI-co-incubated, $^{\#}P < 0{\cdot}001$ *versus* untreated. DFO, deferoxamine; DFX, deferasirox; ROS, reactive oxygen species; PBMC, peripheral blood mononuclear cells; HSPC, haematopoietic stem/progenitor cell; DCF MFI, 2′,7′-dichlorodihhyrofluorescein-diacetate-related mean fluorescence intensity; DPI, diphenyleneiodonium; CTRL, control.

SOX2, *SOX17*, *NANOG* and *MYC,* involved in the control of the self-renewal *versus* differentiation balance. The result of the quantitative real-time-PCR analysis (Fig 2A,B) shows that transcription of POU5F1 and *SOX2* was significantly up regulated upon 24-h DFX treatment (DFO treatment had no or negligible effect) and, most notably, this effect was completely abrogated by DPI co-incubation ($P = 0{\cdot}04$ versus DFX). Conversely, the transcript levels of *NANOG* and *MYC* did not result in significant changes following treatment with either of the two iron chelators (data not shown).

Surprisingly, the transcript level of *SOX17*, a specific marker of fetal HSC barely expressed in adult HSCs, was markedly and specifically up regulated in a DPI-sensitive manner (Fig 2C). Consistent with this finding, the protein level of CTNNB1, known to be negatively controlled by *SOX17*, was significantly decreased upon DFX treatment (Fig 2D). Notably, the expression of BMI1, which was reported to be associated with, and regulated by, CTNNB1 (Kirstetter *et al*, 2006; Lv *et al*, 2012) was similarly depressed by DFX (Fig 2E). These effects were fully prevented by DPI and absent upon DFO treatment.

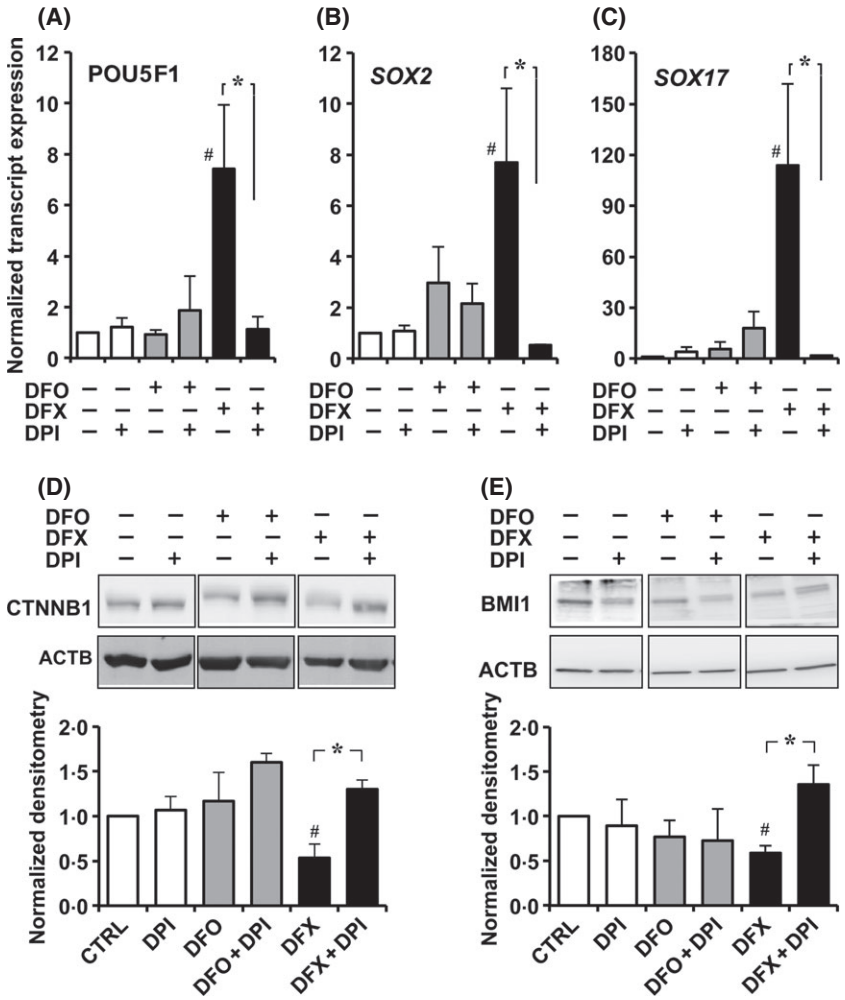

Fig 2. Expression of stemness transcription factors and regulatory proteins in $CD34^+$-HSPCs. (A), (B), (C) Quantitative real-time-polymerase chain reaction analysis of transcripts for POU5F1, *SOX2* and *SOX17* respectively in HSPCs; cells were incubated for 24 h with DFO or DFX ± DPI as in Fig 1. The values are means ± standard error of the mean (SEM) of normalized transcript levels of three independent experiments performed with different preparations of HSPCs isolated from different donors; $^{\#}P < 0{\cdot}001$ *versus* untreated, $^{*}P < 0{\cdot}001$ *versus* DPI-co-incubated. (D), (E) Western blot analysis of CTNNB1 (β-Catenin) and BMI1 in HSPC total protein extracts. Cells were treated with DFO or DFX ± DPI for 24 h as in (A–C); the upper panels show representative immunoblots and the lower histograms the means ± SEM of the normalized densitometric analysis from three independent experiments performed with different preparations of HSPCs isolated from different donors ($n = 3$); $^{\#}P < 0{\cdot}05$ *versus* untreated, $^{*}P < 0{\cdot}01$ *versus* DPI-co-incubated. DFO, deferoxamine; DFX, deferasirox; HSPC, haematopoietic stem/progenitor cell; DPI, diphenyleneiodonium; CTRL, control.

Effect of DFX on differentiation markers of HSPCs

To further support the suggestion of specific DFX-mediated deregulation of haematopoiesis, we analysed the impact of the two iron chelators on the expression of CD34, a commonly used surface marker of HSPCs. The flow-cytometric evaluation of the CD34 distribution in untreated HSPCs persistently indicated the presence of an heterogeneous population with a subset of HSPCs expressing low levels of CD34, probably indicating a pre-committed progenitor compartment (see the shoulder in the distribution curve of untreated HSPC (CTRL) of Fig 3A and Piccoli *et al*, 2005). Treatment of HSPCs with 100 μmol/l DFO for 24 h did not cause any appreciable change in the expression/distribution of CD34. Conversely, treatment of HSPC with DFX under identical conditions resulted in an evident enrichment of $CD34^{Low}$ HSPCs.

The same analysis was extended to the surface markers CD117 and CD71; the former (also termed KIT) is a marker of haematopoietic progenitors that is specifically expressed at high levels in common myeloid progenitor, whereas the latter (also known as transferrin receptor, TFRC) is a highly effective marker for the detection of cells of the erythroid lineage. DFX treatment of HSPCs for 24 h caused a significant down- and up-regulation of CD117 and CD71, respectively (Fig 3B). Conversely, treatment of HSPCs with DFO alone resulted in negligible changes in the expression of the two commitment markers. CD33 and CD19 were barely expressed in untreated HSPCs and treatment for 24 h was ineffective in inducing or modulating their expression (data not shown).

All together the results presented indicate that DFX, but not DFO, caused an unstable state in the cellular physiology of HSPCs, priming them toward erythroid commitment. Most importantly, this effect was evident following only 24 h treatment.

Effect of DFX on mitochondrial distribution in HSPC subsets

As HSC differentiation is also accompanied by an increasing number of mitochondria (Piccoli *et al*, 2013), HSPCs were double-stained with a CD34-specific antibody and

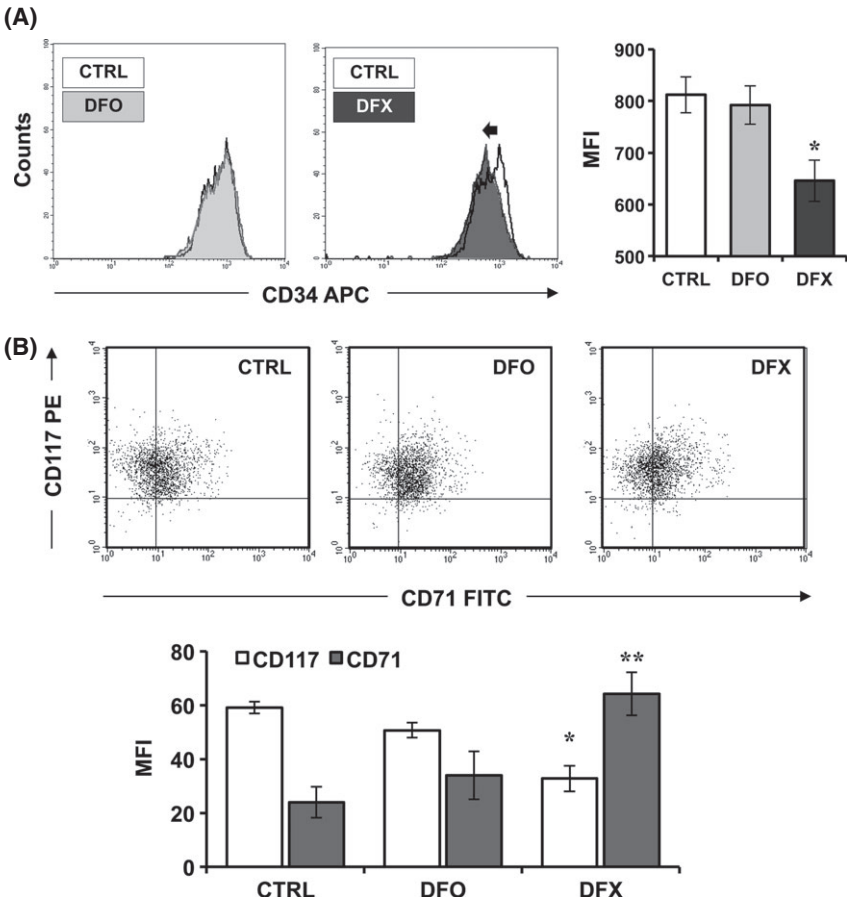

Fig 3. Effect of DFO and DFX on surface markers expression in HSPCs. (A) Representative flow cytometry analysis of CD34 distribution in untreated (CTRL) and 100 μmol/l DFO- or DFX-treated HSPCs (for 24 h), right and middle panel respectively; the small arrow highlights the peak-shift in $CD34^+$-HSPCs following DFX-treatment. The histogram on the left shows the average MFI ± SEM from three independent HSPC preparations from different donors; *$P < 0{\cdot}05$ *versus* CTRL. (B) Representative flow-cytometric two dimensional dot plot of CD117 and CD71 co-staining assessed in untreated and either DFO- or DFX-treated HSPCs as in panel (A). The histogram at the bottom shows the average MFI ± SEM from three independent HSPC preparations from different donors; *$P < 0{\cdot}05$, * $P < 0.01$ *versus* CTRL. See *Methods* for experimental details. DFO, deferoxamine; DFX, deferasirox; HSPC, haematopoietic stem/progenitor cell; MFI, mean fluorescence intensity.

MitoTracker Green, a green-fluorescent probe, which localizes to mitochondria, and assayed by flow cytometry. Figure 4A clearly shows that the subset of $CD34^{High}$ HSPC was characterized by a relatively low staining of the mitochondrial tracer when compared with the $CD34^{Low}$ HSPC, which exhibited much higher mitochondria-related staining. Notably, while DFO treatment did not modify the heterogeneous distribution of mitochondria in the $CD34^+$ HSPC, treatment with DFX under identical conditions (i.e. 100 μmol/l for 24 h) resulted in a striking change of the mitochondria-related fluorescence profile with a significant enhancement in the $CD34^{Low}$ HSPC subset and a marked reduction in $CD34^{High}$ HSPC (Fig 4B,C). Although MitoTracker green was reported to stain mitochondria regardless of their capacity to generate a respiration-dependent membrane potential, we tested the impact of DFX on the respiratory activity of isolated mitochondria without attaining significant effects with up to 200 μmol/l of the drug (data not shown).

Discussion

Several clinical reports have described an unexpected effect of iron chelators, such as improvement in haemoglobin levels, in MDS patients. Among those, DFX induces a similar improvement more rapidly. Although this evidence would infer an effect of the drug on the haematopoietic compartment, no investigation that elucidated a possible molecular mechanism has been reported. The present study demonstrated that DFX treatment is able to prime, *in vitro,* adult HSPC differentiation towards the erythroid lineage and that this process might be linked to ROS production through the selective modulation of defined molecular targets.

Reactive oxygen species represent a critical point in the pathogenesis of MDS (Omidvar *et al*, 2007) as well as in disease progression in myeloid malignancies (Koptyra *et al*, 2006; Rassool *et al*, 2007). Given that an excess of transfusional iron results in marked increased oxidative stress (Hershko *et al*, 1998), iron chelation would be expected to lower cellular ROS levels. Consistent with this hypothesis, several clinical trials indicated that DFX treatment could reduce oxidative stress parameters in transfusion-dependent patients (Gattermann *et al*, 2010; Ghoti *et al*, 2010; Fibach & Rachmilewitz, 2012; Saigo *et al*, 2013). Conversely, Messa *et al* (2010) demonstrated that DFX acts as a potent nuclear factor-kB inhibitor, independent of possible ROS scavenging activity both *in vitro* in leukaemia cell lines and *in vivo* in peripheral blood samples of MDS. On the other hand, *in vitro* experiments performed on progenitor cells $CD34^+$ isolated from MDS patients show that DFX exposure induces ROS production in a dose- and time-dependent fashion (Pullarkat *et al*, 2012).

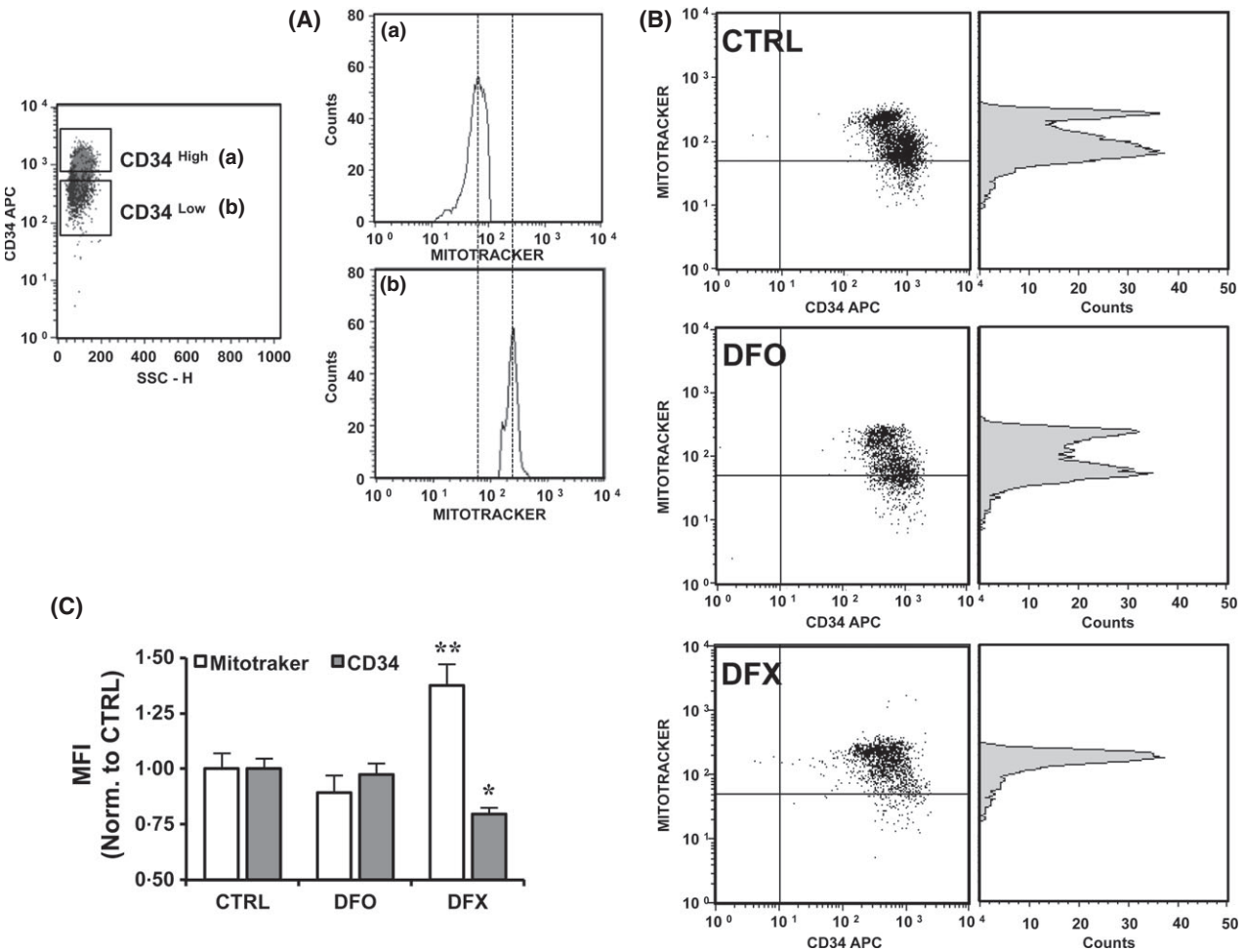

Fig 4. Effect of DFO and DFX on the mitochondrial distribution in $CD34^+$-HSPCs (A) Mito Tracker green distribution in HSPC subsets. The cloud diagram shows a representative gating of $CD34^{High}$ and $CD34^{Low}$ of untreated HSPC cells. The mean intensity signal of the cellular fluorescence distribution in gated $CD34^{High}$ (a) and $CD34^{Low}$ (b) is shown on the right. (B) Representative flow-cytometric two-dimensional dot plot of untreated (CTRL) and 100 μmol/l DFO- or DFX-treated HSPCs co-stained for Mito Tracker green and CD34; panels on the right display the Mito Tracker green distributions. (C) Quantitative analysis of the MitoTraker-and CD34-related MFI is shown as average (±SEM) of three independent HSPC preparations from different donors treated as in panel (B); $*P < 0{\cdot}05$ and $**P < 0{\cdot}01$ *versus* CTRL. See *Methods* for experimental details. DFO, deferoxamine; DFX, deferasirox; HSPC, haematopoietic stem/progenitor cell; MFI, mean fluorescence intensity; Norm., normalized.

In keeping with the clinical heterogeneity and the biological complexity of MDS patients, we thought that this would have introduced a bias in the identification of the DFX mechanism of action. Therefore we used adult haematopoietic cells isolated from healthy subjects as an experimental model. Our preliminary experiments performed on PBMCs to determine the best experimental conditions clearly indicated a dose-dependent, DFX-mediated ROS induction without affecting cell viability. Moreover, ROS up-regulation, observed after 24 h of DFX treatment, but already manifest after 4 h, was prevented by DPI at 20 μmol/l, a concentration inhibiting the main sources of ROS, such as the respiratory chain complexes containing flavinic groups and the NADPH-oxidases (Piccoli *et al*, 2007a). These data represent the first observation that DFX induces ROS in adult healthy terminally differentiated blood cells and unpublished data obtained in our laboratory led us to suppose that this ability is not limited to haematopoietic cells.

Given that an extensive body of literature highlights the importance of ROS as signal molecules in pre-commitment phase of adult stem cells (Piccoli *et al*, 2007b; Sardina *et al*, 2012), our study undertook the further step of investigating the consequence of DFX treatment directly on HSPCs. Pullarkat *et al* (2012) demonstrated a dose-dependent increase of ROS levels in $CD34^+$ cells isolated from MDS during *in vitro* culture, compared to progenitors isolated from human umbilical cord blood. We confirmed the ability of DFX in inducing ROS production also in healthy HSPCs after 24 h of treatment, independent of its iron chelating properties. In fact, the other iron chelator DFO, tested at the same concentration of DFX, did not significantly affect ROS levels compared to untreated HSPCs. The different iron chelating

activity of the two drugs (i.e. 2:1 vs. 1:1 chelator:iron ratio for DFX and DFO, respectively) would imply a less efficient complexing activity of DFX. However, it must be pointed out that both chelators were in large excess with respect to the estimated total iron content in 10% fetal bovine serum-supplemented RPMI [i.e. 100 μmol/l vs. about 5 μmol/l total iron (Kakuta *et al*, 1997)]. In any case, this argument reinforces our belief that the observed effect of DFX is largely independent, at least *in vitro*, of its iron-chelating activity.

The preserved cell viability demonstrated in our experiments, as well as a recent piece of evidence indicating a reduction of oxidative DNA damage after DFX in *in vitro* treatment (Kikuchi *et al*, 2012), suggested that DFX was able to induce intracellular redox signalling without provoking their known deleterious effects. We sought therefore to identify molecular targets selectively modulated by DFX through ROS signalling activation. In addition to the well-known markers used to monitor HSPC self renewal/differentiation processes, there are transcription factors, such as POU5F1 *SOX2*, *NANOG* and *MYC*, that are commonly applied to identify embryonic stem cells (Calloni *et al*, 2013). An extensive body of literature reports how ectopic overexpression of these genes in several cellular types and species can induce differentiated cells to reprogramme their morphological and functional characteristics, leading to induced pluripotent stem cells (Takahashi & Yamanaka, 2006; Takahashi *et al*, 2007; Palma *et al*, 2013). However, their endogenous presence and role in adult stem cells, particularly HSCs, remain to be elucidated. Tsai *et al* (2012), for instance, had already demonstrated that POU5F1 and *NANOG* overexpression in adult mesenchymal stem cells is not only essential for the maintenance of pluripotency, but also increase cell proliferation rate and differentiation potential. Our analysis showed that DFX is able to induce a seemingly ROS dependent up-regulation of POU5F1 and *SOX2* gene expression. In contrast, *NANOG* and *MYC* gene expression did not show selective DFX-mediated regulation.

SOX17 is known to be not expressed by adult stem cells. It plays a pivotal role in fetal haematopoiesis, especially in the maintenance of fetal and neonatal HSCs (Clarke *et al*, 2013). Most notably, overexpression of *SOX17* is sufficient to confer self-renewal potential and fetal stem cell characteristics to haematopoietic progenitors (He *et al*, 2011). Moreover, and counter-intuitively, it has been reported by genome-wide mapping of *SOX17*-binding sites that *SOX17* activates the transcription of key regulator genes for erythrocyte differentiation directly (Nakajima-Takagi *et al*, 2013).

Studies performed in other cellular models indicate *SOX17* as a transcription factor that induces cell differentiation through inhibition of canonical WNT/CTNNB1 signalling (Chen *et al*, 2013), which is relevant for adult HSC, as appropriate CTNNB1 levels guarantee the correct haematopoietic self/renewal and differentiation (Zon, 2008). Our results demonstrate that, as expected, untreated HSPCs did not express *SOX17* mRNA, while DFX-treated samples induced a robust expression of *SOX17* transcript accompanied with a significant down-regulation of CTNNB1 protein expression. Interestingly, DPI co-incubation with DFX restored basal condition. We also observed a significant ROS-dependent down-regulation of BMI1 protein levels in HSPCs treated by DFX. In this setting, BMI1 represents another regulatory protein required to control the adult self-renewing HSC (Park *et al*, 2003), recently indicated as a molecular marker for predicting the prognosis and progression of MDS patients (Mihara *et al*, 2006).

Taken together, these findings support the hypothesis that DFX is able to generate an unstable state of HSPCs, activating specific redox sensitive factors of the transcriptional and translational machinery controlling the balance between self-renewal and differentiation. The faint modulation observed by DFO-treatment would suggest a probable iron chelation-unrelated mechanism by which DFX exerts its action.

It is known that mitochondrial activity may also represent an important regulatory mechanism that helps to determine stem cell fate (Rehman, 2010). In particular, uncommitted stem cells are characterized by lower mitochondrial mass, which changes in HSCs during the early steps of the proliferation/differentiation process. We previously showed an inverse correlation between CD34 expression and mitochondrial content in HSCs (Piccoli *et al*, 2005) and these findings are confirmed here, as mitochondria labelling of untreated HSPCs revealed the presence of $CD34^{High}$ expressing cells, characterized by lower mitochondria content, and $CD34^{Low}$ HSPCs, featuring higher mitochondrial mass. Interestingly, our study demonstrated that DFX was able to increase mitochondrial content together with CD34 down-regulation. It is accepted that the regulatory role of mitochondria is achieved through the controlled release of multiple signalling molecules, also including ROS (McBride *et al*, 2006). Preliminary imaging analysis of DFX-treated HSPCs with ROS-sensitive probes resulted in a peculiar dotted signal, resembling the mitochondrial network (C.P. unpublished data). Although further development is needed, these observations support our proposal that DFX enables reprogramming of HSCs in a process involving mitochondrial ROS.

The last aim of our study was therefore to understand whether all these intracellular modifications were able to modulate HSPC surface molecules. CD117 down-regulation, together with the CD34 reduction already observed, confirmed an early loss of stemness. The contextual increase of the transferrin receptor (CD71, TFRC), not accompanied by any modulation in CD33 or CD19 expression, used as myeloid and lymphoid markers respectively, let us to conclude that DFX treatment induces a specific erythroid differentiation of HSPCs. Taoka *et al* (2012) have already showed that iron overload impairs erythroblast differentiation of human haematopoietic progenitors cultured to produce erythroid burst-forming unit *in vitro*. Moreover, Hartmann *et al* (2013) had demonstrated an iron overload-dependent suppression of erythropoiesis in MDS patients. Our findings demonstrate the ability of DFX to directly

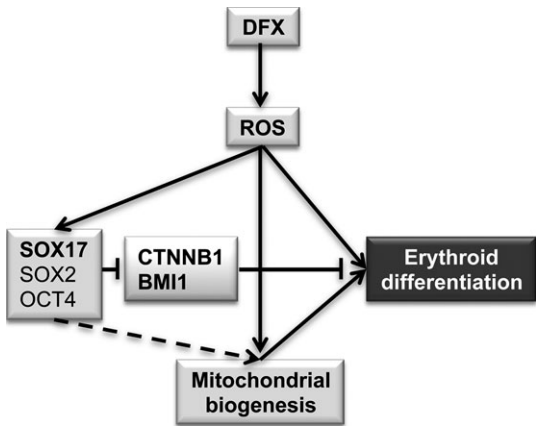

Fig 5. Suggested mechanism of action of DFX in HSPCs. The scheme shows the possible interplay between DFX-mediated ROS production, activation of transcription/regulatory factors, mitochondrial biogenesis and erythroid commitment in HSPCs as suggested by the present study; see *Discussion* for details. DFO, deferoxamine; DFX, deferasirox; ROS, reactive oxygen species.

address HSPCs towards the erythroid lineage, which is a peculiar ability of this drug as DFO did not induce any modulation of the evaluated markers. The scheme shown in Fig 5 summarizes the possible interplay in HSPCs between DFX-mediated ROS production stimulation, activation of specific transcription factors, mitochondrial biogenesis and erythroid commitment.

In conclusion, our results show for the first time, that DFX treatment, probably independent of its iron-chelating property, is able to induce ROS production that, in turn, influences key factors involved in self-renewal/differentiation of HSC. These orchestrated events, culminating in an evident erythroid commitment, could explain the beneficial effects on haematopoiesis observed in some MDS patients treated with DFX. Moreover, we may speculate that, in addition to the DFX-mediated pathway unveiled in this *in vitro* study, other additional DFX-dependent effects might be elicited in the *in vivo* setting. The relatively small proportion of MDS patients responding to DFX-treatment with increased Hb level might rely on the wide clinical and biological heterogeneity of MDS. Our study suggests the possibility of using cytofluorimetric analysis for the detection of potentially responsive patients.

Interestingly, it has been recently reported that oral administration of DFX resulted in haematopoietic recovery in all eight patients submitted to allogeneic HSC transplantation who were heavily transfused before transplant (Visani *et al*, 2014). In this scenario, even if needing confirmation in the clinical setting, the modulation of ROS, because of their ability to restore the haematopoietic function, could be taken into account as potential further pharmacological targets for MDS treatment or, more generally, to improve transfusion-related complications. The results of the present study will be further advanced if the observations attained on normal HSPCs can be recapitulated in those of DFX-treated haematological patients.

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Authorship contribution

Contribution: T.T. planned, performed and coordinated the experiments and interpreted the results; F.A., C.M., V.R., R.S., I.L., F.D. performed the experiments and interpreted the results; F.F., M.D. provided biological samples and background information; P.M. provided background information; N.C. helped design the experiments, interpreted the results, provided background information, wrote and edited the manuscript; C.P. conceived and coordinated the study, planned and performed the experiments, interpreted the results, wrote and edited the manuscript.

Competing interests

The authors have no competing interests.

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