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OPINION

ARTICLE SERIES: STEM CELLS

Reliability of ROS and RNS detection in hematopoietic stem cells – potential issues with probes and target cell population

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ABSTRACT

Many studies have provided evidence for the crucial role of the reactive oxygen species (ROS) and reactive nitrogen species (RNS) in the regulation of differentiation and/or self-renewal, and the balance between quiescence and proliferation of hematopoietic stem cells (HSCs). Several metabolic regulators have been implicated in the maintenance of HSC redox homeostasis; however, the mechanisms that are regulated by ROS and RNS, as well as their downstream signaling are still elusive. This is partially owing to a lack of suitable methods that allow unequivocal and specific detection of ROS and RNS. In this Opinion, we first discuss the limitations of the commonly used techniques for detection of ROS and RNS, and the problem of heterogeneity of the cell population used in redox studies, which, together, can result in inaccurate conclusions regarding the redox biology of HSCs. We then propose approaches that are based on single-cell analysis followed by a functional test to examine ROS and RNS levels specifically in HSCs, as well as methods that might be used *in vivo* to overcome these drawbacks, and provide a better understanding of ROS and RNS function in stem cells.

KEY WORDS: Hematopoietic stem cells, Reactive nitrogen species, Reactive oxygen species, Self-renewal

Introduction

Recent studies have revealed that a population of hematopoietic stem cells (HSCs) that resides in the hypoxic niche and mostly relies on anaerobic glycolysis is highly sensitive to oxidative stress (Simsek et al., 2010; Suda et al., 2011; Unwin et al., 2006). HSCs, and stem cells in general (Han et al., 2008), are highly vulnerable to increased levels of reactive oxygen species (ROS), whereas committed hematopoietic progenitor cells (HPCs) are not (Chen et al., 2008b; Ito et al., 2004; Jang and Sharkis, 2007). Nevertheless, an appropriate production of ROS is required for HSC functions, such as mobilization, survival, differentiation and proliferation (Guzy and Schumacker, 2006; Hole et al., 2010; Hosokawa et al., 2007; Jang and Sharkis, 2007; Sattler et al., 1999; Tesio et al., 2011; Wang et al., 2013). Furthermore, ROS modify gene expression and the activity of key metabolic regulators, thereby determining specific metabolic patterns and cell-fate decision (Bigarella et al., 2014). However, an excess of ROS is associated with DNA damage, lipid peroxidation, senescence and apoptosis (Naka et al., 2008; Navarro-Yepes et al., 2014; Shao et al., 2011). Furthermore, elevated ROS levels and accumulation of damaged DNA in human cord blood $Lin^{-}CD34^{+}38^{-}$ cells (enriched in HSCs) result in a reduction of their capacity to reconstitute hematopoiesis in immunosuppressed mice (Yahata et al., 2011). In addition to ROS, reactive nitrogen species (RNS) are integral to the redox status of the cell; ROS and RNS have overlapping functions and also affect each other's functions (Nathan and Cunningham-Bussel, 2013). Considering the role of RNS, nitric oxide (NO•) inhibits proliferation of HSCs and HPCs, and blocks their progression through the cell cycle, as well as induces apoptosis of $CD34^{+}$ bone marrow (BM) cells *in vitro* (Reykdal et al., 1999). Furthermore, inhibition of nitric oxide synthase (NOS) increases the number of stem cells in the BM and the longevity of hematopoiesis in continuous BM cultures (Epperly et al., 2007; Michurina et al., 2004). However, compared with the work on ROS, there are considerably fewer studies that address RNS-mediated effects on HSCs and the signaling networks they participate in (Aleksinskaya et al., 2013; Bonafê et al., 2015; Nogueira-Pedro et al., 2014).

It has proven difficult to identify the specific ROS and RNS species that affect the functions of HSCs. This is partly due to a lack of suitable methods and chemical probes that allow the unequivocal distinction between different ROS and RNS, and to detect the complex intracellular chemistry of these diffusible and short-lived species. Furthermore, with the exception of a few studies that have been performed by using cell populations that were highly enriched in functional HSCs (Chuikov et al., 2010; Jung et al., 2013; Liu et al., 2009; Taniguchi Ishikawa et al., 2012), most studies that describe the role or consequence of ROS levels in redox measurements of HSCs were performed in heterogeneous cell populations that consist mainly of committed progenitor cells and only a small portion of functional HSCs (Table 1). In this Opinion article, we discuss how these limitations can lead to incorrect interpretation of data. Moreover, on the basis of recent advances in research, we present strategies that might enable to accurately detect ROS and RNS in HSCs and, thus, yield a better interpretation of the obtained results. We believe that these methods might result in important progress in our understanding of the redox regulation of stem cell physiology.

ROS and RNS

The biochemical pathways in aerobic organisms that utilize O_2 continuously generate the metabolic by-products ROS and RNS. Thus, aerobes have developed physiological mechanisms that enable ROS and RNS to be either detoxified or used as intracellular or intercellular signaling messengers to maintain cellular homeostasis. However, ROS and RNS are not single entities but a broad range of chemically distinct reactive species with diverse biological reactivities (Nathan and Cunningham-Bussel, 2013). There are several intracellular sources of ROS, including the mitochondrial electron transport chain (ETC), the membrane-bound NADPH oxidase (NOX) complex, the endoplasmic reticulum, oxidoreductase enzymes and metal-catalyzed oxidation reactions (Nathan and Cunningham-Bussel, 2013; Wang et al., 2013) (Fig. 1). Leaked electrons from the ETC can be captured by O_2 to form the anion superoxide ($O_2•^{-}$), which – together with (NO•) – initiates the

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Table 1. Overview of studies that have addressed the roles of ROS and RNS in HSC fate

Observations and conclusions	Model	Target cell population	Probe/sensor used	Redox status observed	Reference
Increase of ROS levels in HSCs, resulting in impaired reconstitutive capacity and/or self-renewal	*Atm*$^{-/-}$ mice	LSK	$DCFH_2$-DA	Cellular ROS production	Ito et al., 2004, 2006
	FoxO 1,2,3-deficient mice	LSK	$DCFH_2$-DA	Cellular ROS production	Tothova et al., 2007; Miyamoto et al., 2007
	Bmi1$^{-/-}$ mice	LSKCD150$^+$ CD48$^-$CD41$^-$	$DCFH_2$-DA; MitoSOX	Cellular ROS production; mitochondrial O_2^- production	Liu et al., 2009
	BID^{AA} mice	CD34$^-$LSK	MitoSOX	Mitochondrial O_2^- production	Maryanovich et al., 2012
	CBCD34$^+$sh HIF1/2	Lin-CD34$^+$ CD38$^-$	CellROX	Cellular ROS production	Rouault-Pierre et al., 2013
Increase of ROS in HSCs, resulting in their exhaustion and/or senescence	*Mdm2*$^{-/-}$ mice	LSK	$DCFH_2$-DA	Cellular ROS production	Abbas et al., 2010
	Prdm1-deficient mice	CD150$^+$CD48$^-$CD41$^-$SK	$DCFH_2$-DA	Cellular ROS production	Chuikov et al., 2010
	Irradiated murine BM	LSK	$DCFH_2$-DA; DHR; HE	Cellular ROS production	Wang et al., 2010
	Txnip$^{-/-}$ mice	LSKCD150$^+$ CD48$^-$CD41$^-$	$DCFH_2$-DA; HE	Cellular ROS production	Jung et al., 2013
	Connexin 43-deficient mice	Lin$^-$CD150$^+$ CD48$^-$CD41$^-$	$DCFH_2$-DA; HE; MitoSOX	Cellular H_2O_2 and O_2• production; mitochondrial O_2^- production	Taniguchi Ishikawa et al., 2012
	Irradiated murine BM	CD34$^+$ CD133$^+$	$DCFH_2$-DA	Cellular ROS production	Shao et al., 2014
	P2ry14$^{-/-}$ mice	LSK	MitoSOX	Cellular ROS production	Cho et al., 2014
HSCs are enriched in the 'ROS low' fraction	Murine BM	Lin$^-$	$DCFH_2$-DA	Cellular ROS production	Jang and Sharkis, 2007
HSCs produce constitutively NADPH oxidase- generated ROS	G-CSF-mobilized CD34$^+$ cells	CD34$^+$	$DCFH_2$-DA	Cellular H_2O_2 production	Piccoli et al., 2005, 2007b
Circulating HSCs express HIF-1 that is stabilized by ROS	G-CSF-mobilized CD34$^+$ cells	CD34$^+$	$DCFH_2$-DA	Cellular ROS production	Piccoli et al., 2007a
Diminished NOX complex activity and ROS content in HPCs and HSCs at low [O_2]	CB	CD34$^+$	$DCFH_2$-DA	Cellular ROS production	Fan et al., 2007; Hao et al., 2011
Suppression of ROS by FoxO3 in HSCs is partly mediated via ATM	*FoxO3*$^{-/-}$ mice	LSK	$DCFH_2$-DA	Cellular ROS production	Yalcin et al., 2008
Decrease of ROS in HSCs; resulting in increased quiescence	*Akt1/2*-deficient mice	LSK	$DCFH_2$-DA	Cellular ROS production	Juntilla et al., 2010
O_2• and H_2O_2 production increase in HPCs, partly with help of the NOX complex	hBM CD34$^+$ expressing RAS	CD34$^+$	$DCFH_2$-DA Amplex Red; EPR, Diogen MitoSOX	Specific ROS detection	Hole et al., 2010
c-Met activity promotes BM mobilization of HPCs via ROS signaling	Murine G-CSF mobilization	BM	HE	Cellular ROS production	Tesio et al., 2011
FLT3-driven H_2O_2 production in AML is mediated by p22phox	hAML cell line FLT3 -ITD	hAML cells	PO1; MitoPY1	Mitochondrial H_2O_2 production	Woolley et al., 2012
Increase of ROS in HPCs not linked with mitochondrial O_2^-	Irradiated CB CD34$^+$ cells	CD34$^+$	$DCFH_2$-DA; MitoSOX	Cellular ROS production; mitochondrial O_2^- production	Yamaguchi and Kashiwakura, 2013

Continued

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Table 1. Continued

Observations and conclusions	Model	Target cell population	Probe/sensor used	Redox status observed	Reference
Irradiation increases NO• content in BM stromal cells; NO• induces ROS level increase in BM	Irradiated murine BM stroma	BM stroma	DFA-2D	Cellular NO• production	Gorbunov et al., 2000
	Murine BM	BM, c-kit$^+$	DCFH$_2$-DA; HE	Cellular ROS production; mitochondrial O_2^- production	Nogueira-Pedro et al., 2014
Hypoxia/Hypercapnia improve the maintenance of CD34$^+$ despite production of ROS	CB CD34$^+$ preserved at 4°C	CB CD34$^+$	DCFH$_2$-DA; EPR; Amplex Red	Cellular ROS, extracellular H_2O_2 and O_2• generation	Vlaski et al., 2014

BM, bone marrow; CB, cord blood; DCFH$_2$-DA, dihydro dichlorofluorescein diacetate; DHR, dihydrorhodamine; DFA, diaminofluorescein; EPR, electron paramagnetic resonance; G-CSF, granulocyte-colony-stimulating factor; HE, hydroethidine; HIF-1, hypoxia-inducible factor-1; PO1, Peroxy Orange 1; MitoPY1, Mitochondria Peroxy Yellow 1.

production of ROS and RNS. O_2•$^-$ can be rapidly converted into hydrogen peroxide (H_2O_2) by superoxide dismutases (SODs) or can react with NO• to form peroxynitrite ($ONOO^-$). H_2O_2 can be converted into the hydroxyl radical (•HO) in the presence of metal ions (Fe^{2+} or Cu^{2+}) in the Fenton reaction. In the presence of CO_2, which is formed during multiple intracellular enzymatic reactions, $ONOO^-$ gives rise to the RNS nitrosoperoxocarboxylate ($ONO_2CO_2^-$), which decomposes rapidly into the nitrogen dioxide (NO_2•) and carbonate (CO_3•$^-$) radicals. The vast majority of biological reactions of $ONO_2CO_2^-$, including those with oxidant-sensitive probes, are mediated by NO_2• and CO_3•$^-$ (Ferrer-Sueta and Radi, 2009). In the presence of chloride or thiocyanate, myeloperoxidase catalyzes the conversion of H_2O_2 into the oxidants hypochlorous acid (HOCl) or hypothiocyanous acid (HOSCN), respectively (Wardman, 2007; Winterbourn, 2014). The cellular levels of these oxidants are determined by the balance between their generation and capture by a number of antioxidant enzymes (e.g. catalases, peroxiredoxins, thioredoxins, thioredoxin reductases, and methionine sulphoxide reductases) (Nathan and Cunningham-Bussel, 2013). In addition, important redox cellular

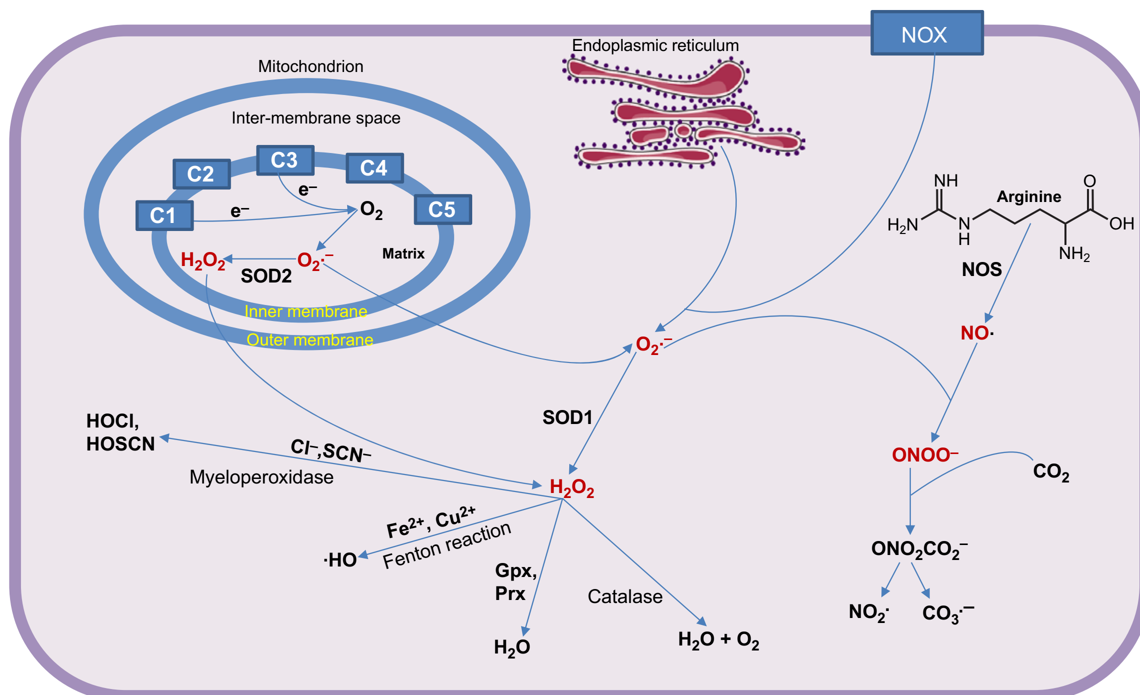

Fig. 1. The cellular ROS and RNS networks. Leaked electrons from the mitochondria electro-transport chain can be captured by O_2, forming anion superoxide (O_2•$^-$) that, together with nitric oxide (NO•), initiates production of ROS and RNS. O_2•$^-$ can be generated by the membrane-bound NADPH oxidase (NOX) complex, the endoplasmic reticulum or the mitochondrial electron transport chain (ETC). Once formed, O_2•$^-$ is rapidly converted into hydrogen peroxide (H_2O_2) by superoxide dismutase 1 (SOD1) in the cytoplasm and SOD2 in mitochondria or reacts with NO• to form peroxynitrite ($ONOO^-$). H_2O_2 can be converted into the hydroxyl radical (•HO) in the presence of metal ions (Fe^{2+} or Cu^{2+}) in the Fenton reaction. In the presence of CO_2, $ONOO^-$ forms the RNS nitrosoperoxocarboxylate ($ONO_2CO_2^-$) that degrades rapidly into a nitrogen dioxide radical (NO_2•) and a carbonate radical (CO_3•$^-$). In the presence of chloride or thiocyanate (Cl^- or SCN^-), myeloperoxidase catalyzes the conversion of H_2O_2 to the oxidants hypochlorous (HOCl) or hypothiocyanous acid (HOSCN), respectively. Catalase, glutathione peroxidase (Gpx) or peroxiredoxin (Prx) enzymes decompose H_2O_2 to H_2O. C1–C5, respiratory chain complexes (C) 1–5. ROS and RNS suggested to be involved in HSC fate are shown in red.

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regulators are enzymes of the glutathione redox cycle, that detoxify H_2O_2 by using the reduced glutathione (GSH) (i.e. glutathione peroxidase) and regenerate oxidized glutathione (GSSG) to reduced glutathione (glutathione reductase) (Nathan and Cunningham-Bussel, 2013).

ROS and RNS have distinct chemical properties that result from differences in their reactivity, half-live and interaction with lipid moieties (D'Autréaux and Toledano, 2007). A selective reactivity with biological targets is the basis for the specific signaling pathways that are induced by individual ROS and RNS (D'Autréaux and Toledano, 2007). For instance, $O_2•^-$ preferentially reacts with proteins that contain iron–sulphur [Fe–S] clusters, whereas the uncharged H_2O_2 does not react with these proteins. Instead, H_2O_2 preferentially reacts with cysteine thiol groups that are present in the catalytic centers of enzymes. Moreover, not all cysteine residues are equally able to be oxidized by H_2O_2; this provides specificity of signaling induced by this ROS. NO• interacts with proteins that contain Fe–S clusters, heme (Cooper, 1999) or specific cysteine thiol groups (Marozkina and Gaston, 2012). The reactions mentioned above induce allosteric or conformational changes in target proteins (Bigarella et al., 2014; Finkel, 2011). These changes affect the function of proteins and their interaction with other proteins, and so can trigger distinct signaling events (D'Autréaux and Toledano, 2007; Stamler et al., 1992).

Redox-regulatory molecules implicated in HSC functions

Several metabolic regulators and signaling pathways have been implicated in the maintenance of the redox homeostasis of HSCs. Activities of these regulators predispose HSCs to certain cell fates as summarized in Table 1.

It must be noted that the definition of an HSC is functional, based on the self-renewal capacity and differentiation potential of the cell. Unlike committed progenitors, HSCs are rare (1 in 10,000 within adult bone marrow cells), multipotent, self-renewing cells with a remarkable regenerative capacity that enables these cells to sustain hematopoiesis over a lifetime. As stated by John Dick: "The only conclusive method to assay stem cells is to follow their ability to repopulate conditioned recipients…" (Dick et al., 1997). HSCs can be identified either by limiting dilution or by single-cell transplantation assays that are based on the capacity of HSCs to hematopoietically reconstitute. For detection of human HSCs, immunosuppressed mice are typically used as recipients and their fate is followed up over 12 to 16 weeks (Ivanovic et al., 2011; Szilvassy et al., 1990; Till et al., 1964). A single HSC is sufficient for successful long-term hematopoietic reconstitution of an immunosuppressed mouse (Osawa et al., 1996).

The first indication that ROS are important in stem cells came from the work of Ito and co-workers (Ito et al., 2004). They demonstrated increased ROS levels in HSC-enriched populations after deletion of the ataxia telangiectasia mutated (ATM) gene in mice ($Atm^{-/-}$ mice) (Ito et al., 2004). These results were obtained in a phenotypically defined cell population that was Lineage (Lin^-, 'L') negative, stem cell antigen (Sca-1, 'S') positive, and c-kit (CD117, 'K') positive. This so-called LSK cell population contains at best ~30% of 'true' HSCs or multipotent progenitors (MPPs) (Oguro et al., 2013). However, often <10% of LSK cells have real HSC potential based on *in vivo* long term-reconstitution capacity (Osawa et al., 1996); instead, the vast majority of these cells are multipotent and committed progenitors (Bryder et al., 2006). Furthermore, the LSK population can be subdivided into fractions based on the expression of SLAM family markers, CD150 (Slamf1) and CD48 (Slamf2): HSCs are particularly enriched in the $CD150^+CD48^-$LSK population, MPPs in the $CD150^-CD48^-$LSK population, whereas $CD150^-CD48^+$LSK and $CD150^+CD48^+$LSK cells contain heterogeneous committed progenitors (Kiel et al., 2005, 2008). The HSCs in the $CD150^+48^-$ population can be further divided into cells that do or do not express the CD34 marker. The most quiescent and potent HSCs were found in the $CD34^-CD150^+CD48^-$ subpopulation (Wilson et al., 2008). Despite the availability of these markers to determine a high enrichment in HSCs and MPPs, the resulting populations remain functionally heterogeneous (Oguro et al., 2013).

In *Atm*$^{-/-}$ mice, an increase in the levels of ROS coincides with the loss of HSC activity despite the maintenance of a phenotypically-defined LSK population (Ito et al., 2004). The underlying mechanisms were shown to involve ROS-mediated activation of p38 mitogen-activated protein kinases (MAPK11 and MAPK14), which upregulates the cyclin-dependent kinase inhibitors $p16^{Ink4a}$ and $p19^{Arf}$. Upregulation of these inhibitors results in a consecutive block of cell-cycle-induced senescence and premature exhaustion of HSCs (Ito et al., 2006). Furthermore, Forkhead box class O (FoxO) transcription factors are crucial regulators of oxidative stress defense and ROS production (Storz, 2011). *FoxO*-deficient mice showed elevated levels of ROS associated with increased cycling and impaired long-term repopulating potential of LSK cells (Miyamoto et al., 2007; Tothova et al., 2007). FoxO-mediated redox regulation in HSCs is achieved partly through the regulation of ATM expression (Yalcin et al., 2008). Treatment of *Atm*$^{-/-}$ or *FoxO*-deficient mice with N-acetyl cysteine (NAC), a scavenger of HOCl, •HO, and H_2O_2 (Aruoma et al., 1989), reverses the ROS-induced defects, suggesting these oxidants have roles in HSCs and HPCs. Recently, it has been shown that ATM is activated directly by oxidative stress, which – in turn – stimulates an anti-oxidative cell response (Ditch and Paull, 2012). One such anti-oxidative protective mechanism is ATM-mediated suppression of the pro-apoptotic protein BID, which is known to act as an inducer of mitochondrial ROS (Maryanovich et al., 2012). The tuberous sclerosis complex (TSC)-mammalian target of rapamycin (mTOR) pathway is a crucial regulator of cell metabolism (Wullschleger et al., 2006). In response to oxidative stress, ATM-dependent inhibition of mTOR maintains quiescence of HSCs by repressing ROS production and mitogenesis (Chen et al., 2008a; Ditch and Paull, 2012). In addition, ROS induce activation of p53, which triggers apoptosis or the expression of the anti-oxidative defense enzymes in LSK cells (Abbas et al., 2010). Hypoxia-inducible factor 1-alpha (HIF1α) has also been implicated in a negative feedback loop of ROS regulation (Honma et al., 2013; Simsek et al., 2010; Takubo et al., 2010); ROS-stabilized HIFα (Piccoli et al., 2007a) induces adaptive metabolic responses that ensure redox homeostasis (Cam et al., 2010), such as inhibition of mTOR signaling and mitochondrial activity (Cam et al., 2010), as well as activation of glycolytic metabolism (Takubo et al., 2013).

Several factors are known to coordinate the balance between stem cell self-renewal and commitment with mitochondrial function. These include members of the polycomb family of proteins, such as B lymphoma Mo-MLV insertion region 1 homolog (Bmi1) and PR-domain-containing 16 (Prdm-16), as well as regulators of the DNA damage response, for example apurinic/apyrimidinic (AP) endonuclease1/redox factor-1 and redox regulator nuclear factor erythroid-2-related factor 2 (Nrf2) (Alfadda and Sallam, 2012; Chuikov et al., 2010; Liu et al., 2009; Wang et al., 2013).

An increase in ROS levels in an LSK population is paralleled by a decline in HSC activity (as measured by reduction of short- and

long-term BM-reconstitution capacity) (Ito et al., 2004; Maryanovich et al., 2012; Miyamoto et al., 2007). However, in view of the fact that the functional HSCs represent often <10% of the LSK population, it is not clear whether ROS levels increase in HSCs themselves. In fact, the exhaustion of HSCs that have been detected in these experiments might originate from a release of ROS by non-HSCs, which represent the vast majority of LSK cells. These released ROS could induce HSC differentiation, senescence and apoptosis and/or necrosis.

Despite progress in understanding their effects on regulation of HSC and HPC functions, the exact ROS- and RNS-mediated mechanisms and their downstream signaling components have not been elucidated. For instance, it is unknown how ROS and RNS mediate cell fate at the molecular level. To fully understand how ROS and RNS contribute to the HSC fate, it is essential to detect these species accurately with regard to their identity, localization, kinetics and quantity because different reactive oxygen and nitrogen species might have very different roles in cellular signaling. For instance, compared with other ROS species, H_2O_2 is relatively stable (with a cellular half-life of ∼1 ms and steady-state levels of 10^{-7} M), diffusible and has selective reactivity (D'Autréaux and Toledano, 2007). These characteristics make it a likely candidate for a signaling molecule. Therefore, it is important to accurately detect this specific molecule without any erroneous contributions of other ROS and RNS species. However, most currently utilized techniques for the detection of ROS and RNS in HSCs and HPCs are not specific for a particular species, and any obtained measurements can be affected by the presence of other ROS or RNS species. Below, we will discuss the limitations and caveats of the commonly used techniques for the analysis of the redox status of stem cells.

Oxidant-sensitive fluorescent probes

The probes most commonly used to detect ROS and RNS are oxidant-sensitive compounds; these probes are often cell-permeable lipophilic esters that undergo intracellular hydrolysis and are retained in the cell, where – upon oxidation – they become fluorescent. Dihydrodichlorofluorescein ($DCFH_2$), hydroethidine (HE), diaminofluorescein (DFA-2), Amplex Red as well as the chemiluminescence detectors, belong in this category (Wardman, 2007; Winterbourn, 2014) (Table 2).

These probes can be oxidized directly by various cellular radical species or by metal-dependent processes, which result in a radical-probe intermediate that is subsequently oxidized into the fluorescent product (Wardman, 2007). The radical-probe intermediate can react with various oxidants or can be captured and scavenged by cellular antioxidants. This means that oxidant-sensitive probes are generally non-specific for a particular oxidant, and their complex chemical behavior makes them unsuitable for mechanistic studies of cellular events that are induced by specific ROS and RNS species (Wardman, 2007; Winterbourn, 2014). Owing to the fact that a positive signal can be generated from non-specific reactions, the reactivity of oxidant-sensitive probes with particular ROS or RNS species should be confirmed by other techniques or by use of appropriate inhibitors (Zielonka et al., 2012b). However, their main advantage is that they are easy to use.

Non-specific detection of ROS and RNS: $DCFH_2$

A common ROS probe is $DCFH_2$ or its widely used esterified cell-permeable diacetate ($DCFH_2$-DA); neither react directly with H_2O_2 to form the fluorescent product dichlorofluorescein (DCF) but require a peroxidase or redox-active metal catalyst (i.e. Fe^{2+}). Indeed, it has been shown that the exposure to H_2O_2 alone is unable to oxidize $DCFH_2$ (Karlsson et al., 2010). This requirement might lead to misinterpretations of data obtained with this probe and this issue has been discussed in detail in a number of reviews (Kalyanaraman et al., 2012; Karlsson et al., 2010; Wardman, 2007; Winterbourn, 2014). For example, a negative signal could be obtained if a metal catalyst is absent, whereas increased oxidation of $DCFH_2$ in the presence of cytochrom *c* – which is released during apoptosis or under conditions of increased iron availability from the lysozyme – could be mistakenly associated with an increased oxidant production, as critically analyzed elsewhere (Burkitt and Wardman, 2001; Karlsson et al., 2010). Furthermore, oxidation of $DCFH_2$ is entirely non-specific and can be achieved by a number of different free radicals – such as derivates of $ONOO^-$, tyrosine, thiols and HOCl as well as by light (Marchesi et al., 1999) – independently of H_2O_2 (Wardman, 2007; Wrona et al., 2005).

Thus, in contrast to what has been concluded in numerous reports with regard to a redox analysis of HSCs and HPCs (Ito et al., 2004, 2006; Miyamoto et al., 2007; Piccoli et al., 2007b, 2005) (Table 1), DCF fluorescence cannot be used as a direct measure of cellular H_2O_2 levels, nor as direct evidence that H_2O_2 is involved in a signaling event (Kalyanaraman et al., 2012; Karlsson et al., 2010; Wardman, 2007; Winterbourn, 2014).

$DCFH_2$ has also been used as a general indicator of the redox status of HSCs and HPCs (Abbas et al., 2010; Chen et al., 2008a; Chuikov et al., 2010; Fan et al., 2007; Hosokawa et al., 2007; Jang and Sharkis, 2007; Jung et al., 2013; Juntilla et al., 2010; Liu et al., 2009; Pazhanisamy et al., 2011; Piccoli et al., 2007a; Shao et al., 2014; Yahata et al., 2011) (Table 1). However, using $DCFH_2$ as a probe for the overall cellular redox status is highly unreliable owing to three limiting factors. First, oxidation of $DCFH_2$ to DCF involves the intermediate radical DCFH• or $DCF•^-$, which – in the presence of O_2 – can generate $O_2•^-$ (Wrona and Wardman, 2006). The subsequent dismutase-mediated oxidation of $O_2•^-$ produces additional H_2O_2, resulting in an artificial amplification of the initial DCF-induced fluorescence signal (Folkes et al., 2009). Second, the efficiency of oxidation of the intermediate $DCFH_2$ probe radical into the final product depends on the environmental oxygen concentration (Wrona and Wardman, 2006). This issue is of particular importance when $DCFH_2$ is used as a probe to study the effect of the O_2 concentration on ROS generation in HSCs and HPCs (Fan et al., 2007; Hao et al., 2011). Third, cellular conditions may also alter the oxidation efficiency of $DCFH_2$. For example, a higher detection sensitivity of $DCFH_2$ in HSCs was reported in the presence of an inhibitor of the P-glycoprotein multidrug-resistant 1 (MDR1) pump (Piccoli et al., 2005); here, an apparent higher sensitivity of $DCFH_2$ might be attributed to reduced expulsion of the probes by MDR1. In this context, it is worth noting that MDR1 is highly expressed in primitive HSCs (defined as cells that can *in vivo* provide long-term bone-marrow hematopoiesis or that can *in vitro* act as long-term culture-initiating cells) (Scharenberg et al., 2002).

Detection of $O_2•^-$: HE, MitoSOX

Hydroethidine (HE) is commonly used for the detection of intracellular $O_2•^-$ in studies of HSCs and HPCs (Jung et al., 2013; Nogueira-Pedro et al., 2014; Tesio et al., 2011; Wang et al., 2010). Although $O_2•^-$ can directly oxidize HE to its radical, the reaction is relatively slow and is more likely to be performed by other cellular oxidants present (Winterbourn, 2014). Once formed, the HE radical combines rapidly with $O_2•^-$ to generate the specific product 2-hydroxyethidium (2-OH-E^+). However, HE can also react with other oxidants (e.g. $ONOO^-$, H_2O_2, •HO) to generate the non-specific product ethidium (E^+) (Kalyanaraman et al., 2012;

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Table 2. Probes and sensors for ROS and RNS detection

Redox sensor	Pros	Cons	Detected ROS/RNS	Cell system
Oxidant-sensitive fluorescent redox probes	Easy to use	Specificity to particular oxidant needs to be confirmed		
$DCFH_2$	Single-cell analysis	Artificial amplification of the fluorescence; non-specific	If used as general ROS indicator, various caveats need to be considered	Intact cells
HE	$O_2•^-$ is capable to directly oxidize HE to generate a specific product, (2-OH-E+); single-cell analysis	Can react with other oxidants forming E+, non-specific product	Intra- and extracellular $O_2•^-$ formation	Intact cells
MitoSOX	Increased affinity to react with $O_2•^-$ compared with HE; single-cell analysis	Same limitations as for HE	Mitochondrial $O_2•^-$ formation	Intact cells
Amplex Red	Amplex-Red-derived radical does not react with O_2 to form additional $O_2•^-$ and H_2O_2	Amplification of light-mediated, artificial fluorescent signal	Highly efficient method to measure the extracellular formation of H_2O_2 in the presence of HRP	Intact cells
DFA	Single-cell analysis	Specificity to generate NO• to be confirmed	NO	Intact cells
Chemiluminescent probes	High sensitivity; quantitative	Same limitations as for oxidant-sensitive fluorescent redox probes	Extracellular $O_2•^-$	Intact cells
EPR	High sensitivity	Problematic to delineate true signals from artifacts	$O_2•^-$, NO•	Cellular and tissue detection
'Non redox' fluorescent boronate probes	Artificial signal generation is avoided; real-time ROS, RNS detection; single-cell analysis	Specificity needs to be confirmed by appropriate inhibitors (e.g. specificity of aromatic boronate)		Intact cells, *in vivo*
PG1, PC1, PCL1			H_2O_2	
CBA			$ONOO^-$	
MitoP1, SHP-Mito, MitoB	Targeted to mitochondria; ratiometric (MitoB)		H_2O_2	
SNAP PG	Organelle targetable	Need for transfection	H_2O_2	
Genetically encoded (single protein) redox sensors	High redox specificity; ratiometric; real-time measurements; easily targeted to subcellular compartments; single-cell analysis	Need for transfection or transduction, generation of transgenetic animals		Intact cells, *in vivo*
roGFP			GSH/GSSG ratio	
Grx-roGFP	High sensitivity		High sensitivity to GSH/GSSG ratio	
Orp1roGFP	Quantitative		H_2O_2	
Hyper	High sensitivity; quantitative	Influence by pH	H_2O_2	
Frex		Influence by pH	NADH	
Peredox	High sensitivity; suitable for mitochondrial NADH detection	Not ratiometric	NADH	
FRET redox sensors	Ratiometric; single-cell analysis	Same as other single-protein genetically encoded sensors; low dynamic range; more or less sensitive to pH		Intact cells
OxyFPET, PerGFP			H_2O_2	
DuoxA1-OxyFRET, DuoxA1-PerFRET			H_2O_2 produced by Duox 1	
Nanoparticle (NP)-based sensors	Ratiometric; optical properties advantageous for *in vivo* studies; great possibility for targeting to subcellular compartments	Disadvantages depend on the type of NP-based sensors described in detail by Uusitalo and Hempel, 2012	Global ROS, H_2O_2, $ONOO^-$	Intact cells, *in vivo*

CBA, Coumarin boronic acid; MitoB, MitoBoronic acid; Duox 1, dual oxidase 1. EPR, electron paramagnetic resonance; FRET, fluorescence resonance energy transfer; Grx-roGFP, glutaredoxin roGFP; GSH/GSSG, reduced glutathione/oxidized gluthation; Orp1roGFP, peroxidase Orp1 fused with roGFP; PG1, Peroxy Green 1; PC1, Peroxy Crimson 1; PCL1, Peroxy-caged luciferin-1; roGFP, reduction-oxidation sensitive green fluorescent protein; SNAP-PG, SNAP Peroxy Green.

Wardman, 2007; Winterbourn, 2014). Although 2-OH-E^+ and E^+ have slightly different fluorescence spectra, they cannot be distinguished easily (Robinson et al., 2006). Therefore, a simple fluorescence assay performed with HE cannot provide sufficient specific information regarding intra- and extracellular $O_2•^-$ formation. Because the fluorescence signal can be generated from

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the non-specific reaction, any specificity of the fluorescent signal for $O_2•^−$ needs to be confirmed with ROS-scavenging enzymes and NOS inhibitors (Zielonka et al., 2012b).

In stem cell redox studies, mitochondrial $O_2•^−$ has been measured by mitochondrial-targeted HE (Mito-HE or MitoSOX) (Cho et al., 2014; Hole et al., 2010; Taniguchi Ishikawa et al., 2012; The et al., 2013). MitoSOX reacts with $O_2•^−$ in a similar manner as HE and, therefore, has the same limitations (Winterbourn, 2014). But as a positively charged molecule, MitoSOX has an increased affinity to react with $O_2•^−$, making the reaction more specific than the HE reaction with $O_2•^−$. Oxidation of MitoSOX by $O_2•^−$ generates the specific product red-fluorescent 2-hydroxymitoethidium (2-OH-Mito-E^+), whereas its reaction with other oxidants results in the non-specific Mito-E (Zielonka and Kalyanaraman, 2010). However, because of the overlap of their fluorescence spectra, MitoSOX cannot be utilized on its own as a reliable indicator of mitochondrial $O_2•^−$ formation (Zielonka and Kalyanaraman, 2010).

Detection of H_2O_2: Amplex Red

The peroxidase substrate Amplex Red is used to detect H_2O_2 that has been extracellularly generated, as well as any that is produced inside the cell and has diffused out (Hole et al., 2010; Skinner et al., 2008; Thompson et al., 2008; Vlaski et al., 2014). Amplex Red can be oxidized to the fluorescent product resorufin by H_2O_2 or other electron oxidants (e.g. $O_2•^−$ and NO•). However, the specificity of Amplex Red for H_2O_2 depends on the presence of sufficient amounts of horseradish peroxidase (HRP). In this case, HRP catalyzes the oxidation of its substrate Amplex Red by H_2O_2 to resorufin in two one-electron oxidation steps (Gorris and Walt, 2009). However, there are some limitations, in particular the light-induced reduction of resorufin in the presence of biological reducing agents, such as NAD(P)H or glutathione, which results in an artificial amplification of the fluorescent signal (Zhao et al., 2011). Thus, in light-protected condition, Amplex Red can be used to specifically measure the extracellular formation of H_2O_2 in the presence of HRP (Kalyanaraman et al., 2012).

Detection of NO•

The probe diaminofluorecscein (DAF-2) and its cell-permeable diacetate (DFA-2DA) are used to monitor the formation of cellular NO• based on the generation of the resulting green fluorescent product DFA-2T (Zielonka et al., 2012b). DAF-2 does not react directly with NO•, but with N_2O_3, which is generated upon NO• oxidation and can be attenuated in the presence of the ascorbic acid (Ye et al., 2004). This method for NO• detection has been used in hematological studies (Čokić et al., 2009; Gorbunov et al., 2000). However, under conditions that generate both NO• and $O_2•^−$, it is not a suitable method to measure NO• selectively, because $O_2•^−$ contributes to the oxidation of DAF-2 into fluorescent DFA-2T. Therefore, NOS inhibitors or ROS-scavengers should be included in the assay in order to discriminate oxidation that is mediated by $O_2•^−$ from that mediated by NO• (Zielonka et al., 2012b).

Chemiluminescent probes

Chemiluminescent assays have been used to detect ROS in HSCs and HPCs (Hole et al., 2010) due to their high sensitivity and the fact they only require a relatively small number of cells (Winterbourn, 2014). However, these assays have the same limitations as oxidant-sensitive fluorescent probes. For instance, an assay based on luminol is unable to discriminate among individual ROS species present in biological systems or provide any mechanistic information (Zielonka et al., 2013). The luminol radical that is generated in the initial, non-specific oxidation can react in numerous interactions that can influence the resulting overall chemiluminescence, as described in detail elsewhere (Wardman, 2007). Luciferin analogues are more specific for the chemiluminescent detection of extracellular $O_2•^−$ (Teranishi, 2007), and this approach could, thus, be used to confirm the production of $O_2•^−$ that has been detected by using an alternative procedure.

'Non-redox' fluorescent probes: boronate probes

Cell-permeable boronate probes contain a blocking group that is attached to the fluorophore. First, an oxidant reacts with and oxidizes the blocking group without changing the oxidation state of the fluorophore. The oxidized form of the blocking group is then released, resulting in fluorescence. These probes are thus referred to as 'non-redox' probes. This mechanism avoids several of the problematic steps that affect the probes discussed above, such as oxidation of the probe, generation of probe-radical intermediates and complex chemical reactions (Winterbourn, 2014). The first application of a non-redox probe was the detection of H_2O_2 in living cells (Dickinson et al., 2013; Lin et al., 2013; Lippert et al., 2011; Woolley et al., 2012). It was later shown that these probes (particularly in the case of aromatic boronate probes) react with $ONOO^−$ much faster than with H_2O_2 (about a million-fold) (Sikora et al., 2009). Thus, the use of the ROS-scavenging enzymes [catalase, polyethylene glycol (PEG) catalase, SOD or PEG-SOD] and a NOS inhibitor ($N^{ω}$-nitro-L-arginine methyl ester), is necessary to distinguish between $ONOO^−$-mediated and H_2O_2-mediated-fluorescence signals (Zielonka et al., 2012a).

Altogether, the studies presented above suggest that currently used techniques for ROS and RNS detection are not sufficiently specific. In addition, with exception of the boronate probe, they cannot be used *in vivo*. In light of these limitations, which are all related to the complexity of cellular redox chemistry, a better approach to examine ROS and RNS *in vitro* and *in vivo* – and specifically in HSCs as opposed to HPC systems – is needed.

Possible single-cell approach for ROS and RNS detection in functional HSCs – determination of HSC redox status *in situ*

A possible assay that could be applied to stem cell biology came from a recent study suggesting that fluorescence-based techniques using HE, boronate probes, DAF-DA, can be used for initial screening before – in a second step – specificity of these probes for certain reactive species is confirmed with NOS inhibitors and ROS-detoxifying enzymes (Zielonka et al., 2012b). It should be noted that there is always uncertainty as to whether the ROS scavengers reach the site of oxidation. Because all these probes allow analysis at single-cell level by flow cytometry, we propose the following procedure to measure ROS and RNS in LSK cell populations that contain HSCs and HPCs (Fig. 2A). A cell population is assayed by using any of these probes (step 1) and specificity of fluorescence signal confirmed before cells are sorted into subpopulations on the basis whether or not they respond to a particular ROS or RNS probe ('ROS/RNS low' or 'ROS/RNS high') (step 2). The following step 3 could then include the sorting of individual cells by their respective phenotypic properties. In the examples shown in Fig. 2A, individual cells within the resulting subpopulations (i.e. ROS/RNS low and ROS/RNS high) are sorted according to whether they express the SLAM family phenotypic markers, CD150 and CD48. The $CD150^+CD48^−LSK$ population that is enriched in HSCs can so be distinguished from a population that contains mainly MPPs ($CD150^−CD48^−LSK$) or HPCs ($CD150^+CD48^+LSKs$) (Oguro

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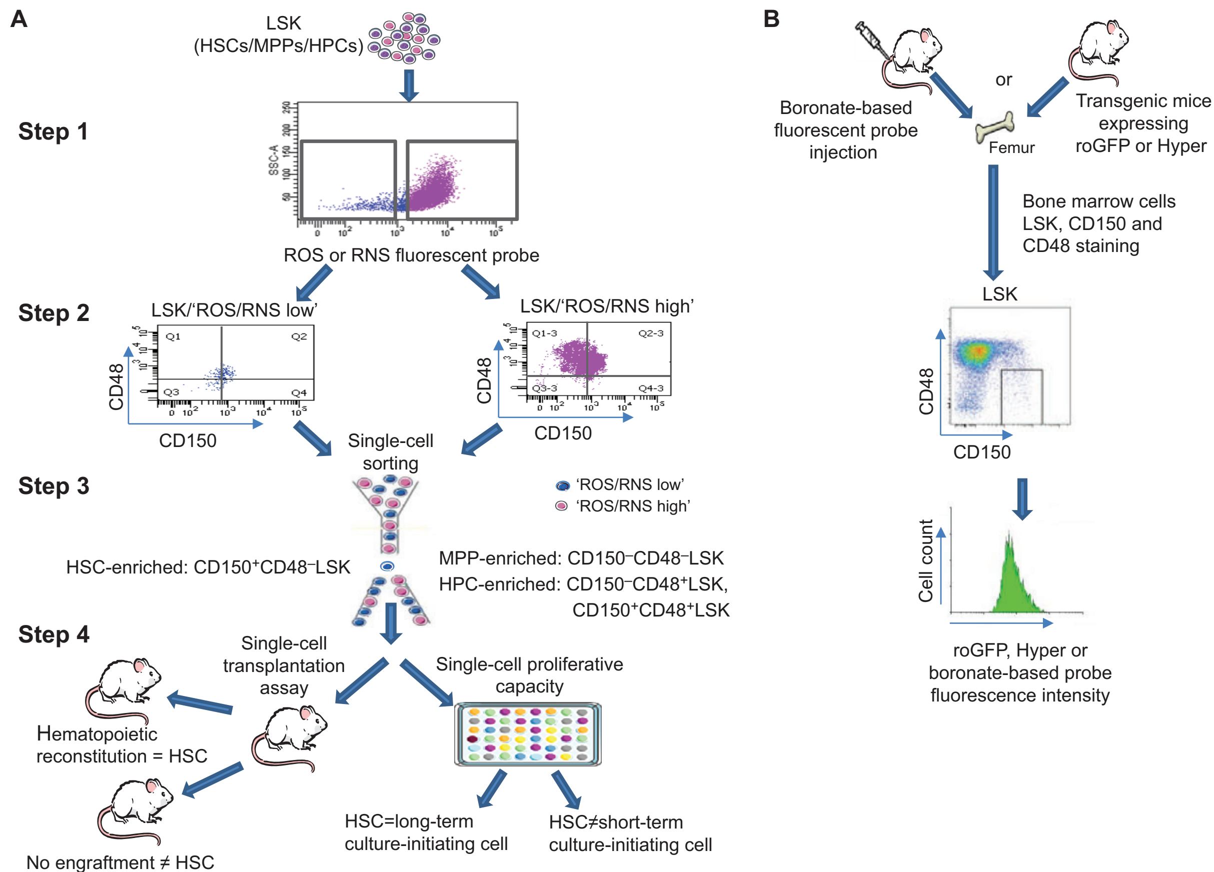

Fig. 2. Possible single-cell approach for ROS and RNS detection in HSCs. (A) Possible experimental single-cell approach to analyse the ROS and RNS content in functional HSCs. A cell population enriched in HSCs (LSK cells in this case) is treated with a fluorescent probe for ROS and RNS (step 1) and examined by flow cytometry to sort the LSK cells into subpopulations on the basis of whether or not they respond to the ROS or RNS probe (i.e. ROS/RNS low or ROS/RNS high) (step 2). Step 3 involves the sorting of individual cells on the basis of their phenotypic properties, such as expression of the SLAM family phenotypic markers CD150 and CD48. The $CD150^{+}CD48^{-}LSK$ subpopulation (enriched in HSCs) can be distinguished from $CD150^{-}CD48^{-}LSK$ cells (enriched in MPPs), $CD150^{+}CD48^{+}LSK$ cells and $CD150^{-}CD48^{+}LSK$ cells that mainly contain HPCs. Subsequently, single cells from the HSC-enriched $CD150^{+}CD48^{-}LSK$ subpopulation are analyzed to identify true HSCs on the basis of their capacity for hematopoietic reconstitution or proliferative potential (step 4). Consecutively, results from the functional assay are correlated with the ROS/RNS status determined in step 2. (B) Possible approach to detect ROS in HSC-enriched cell populations *in situ*. To determine the redox state of a HSC compared with that of HPCs *in situ*, a first step could involve the creation of transgenic mice that express the genetically encoded fluorescent-protein-based redox sensors roGFP or Hyper. The redox state or H_2O_2 levels could then be followed in a sorted, HSC-enriched subpopulation ($CD150^{+}CD48^{-}LSK$ cells). The same strategy could also include the use of boronate-based fluorescent probes that allow to measure changes in H_2O_2 levels in real-time.

et al., 2013). This step could then be followed by the functional analysis of the resulting single cells in order to determine whether they are true HSCs because this cannot be determined with certainty on the basis of surface markers analysis alone (step 4). Such a functional analysis could involve the testing of single cells for their capacity for hematopoietic reconstitution or their proliferative potential (Fig. 2A). This type of approach would not only help to overcome the problem of functional heterogeneity in phenotypically homogeneous HSCs/HPCs populations (such as LSK cells), but also allow to determine the ROS and/or RNS profiles of functional HSC subpopulations.

Furthermore, there are other approaches to determine the redox state of HSCs compared with that of HPCs in their physiological environment. A major breakthrough of ROS detection *in vivo* within cellular and subcellular compartments, as well as *in vitro*, was the development of genetically encoded fluorescent-protein-based redox sensors that are based on the introduction of cysteines into yellow or green fluorescent protein (rxYFP or roGFP) (Dooley et al., 2004; Østergaard et al., 2001). Signal detection is based on the change in the chromophore spectrum upon oxidation or reduction of these cysteines (Lukyanov and Belousov, 2014). The extent of the redox change can be estimated from the relative fluorescence intensity of the two fluorescence excitation maxima of the sensor, making the measurement ratiometric (Lukyanov and Belousov, 2014). These sensors enable specific, real-time, reversible, ratiometric detection of the changes in the redox state (Lukyanov and Belousov, 2014). In addition, these probes enable the specific detection and quantification of H_2O_2 or of the glutathione redox potential (GSH:GSSG ratio) at the single-cell level *in vitro* (Shimi et al., 2011) and *in vivo* (Albrecht et al., 2011; Kojer et al., 2012). For instance, using roGTP transgenic flies, this approach has enabled the *in vivo* mapping of H_2O_2 and of oxidized glutathione during development and aging (Albrecht et al., 2011). Another H_2O_2-sensitive sensor, Hyper, was developed by inserting the circularly permuted YFP (cpYFP) into the regulatory domain of the *E. coli* H_2O_2-sensing protein OxyR (Choi et al., 2001). This probe enabled *in vivo* imaging of H_2O_2 gradients at tissue-scale as well as their quantification (Bilan et al., 2013; Love et al., 2013). Furthermore, this method was used to follow redox changes specifically in Hyper-expressing neutrophils *in vivo* (Pase et al., 2012).

By using these genetically modified approaches, it should be possible to measure the redox state of a HSC *in situ.* For instance, a mouse expressing roGFP or Hyper could be generated, from which HSC-enriched subpopulations are obtained according to the flow cytometry-based sorting procedure outlined above in order to determine and analyze their ROS levels (Fig. 2B). Alternatively, boronate-based fluorescent probes that enable measurement of real-time changes in H_2O_2 in tissue, cells and subcellular compartments could be used to assess the HSC redox status *in situ* (Cochemé et al., 2011; Dickinson et al., 2011; Van de Bittner et al., 2010). Here, boronate probes could be administered to mice via a tail-vein injection before their bone marrow cells are extracted, analyzed and sorted by flow cytometry with respect to their redox status and surface markers (Fig. 2B).

Other promising tools for the ROS and RNS detection in HSCs are fluorescence resonance energy transfer (FRET)-based redox sensors. In FRET, energy is transferred from a light-excited fluorophore (donor) to a longer-wavelength fluorophore (acceptor), whose absorption spectrum overlaps with the emission spectrum of the donor (described in detail by Lukyanov and Belousov, 2014). The main advantage of FRET-based probes lies in the fact that the ratio between the two wavelengths is measured. Unfortunately, these sensors exhibit a low dynamic range and are pH sensitive. Nevertheless, FRET probes have proven very useful and can also be used to probe subcellular compartments or to measure NADPH-oxidase-generated H_2O_2 (Enyedi et al., 2013; Kolossov et al., 2008). However, to date, FRET-based redox sensors have not been used *in vivo*.

It is of particular interest to measure ROS levels (e.g. H_2O_2) under physiological or stress conditions in a hematopoietic niche, as this would help to shed light on the relationship between the state of stem cells and the microenvironment in which they reside. This has been recently achieved for the measurement of the absolute pO_2 concentration in the bone marrow hematopoietic niche by using a metallo-porphyrin-based two-photon-enhanced platinum-porphyrin phosphorescent oxygen-sensitive probe (PtP-C343) (Spencer et al., 2014).

Genetically encoded fluorescent probes or boronate-based fluorescent probes have been used to detect H_2O_2 in various tissues (Bilan et al., 2013; Logan et al., 2014; Love et al., 2013) but not in bone marrow. We believe that their use for H_2O_2 detection in bone marrow should be considered. Another possibility is the use of nanoparticle-based sensors, which have shown to be useful for ROS detection in whole-animal studies (Uusitalo and Hempel, 2012). Nanoparticles can be conjugated with different moieties for their targeting to certain cells or subcellular compartments (Uusitalo and Hempel, 2012). Recently, intravenous injection of specially designed, liver-targeting semiconducting polymer nanoparticles enabled the simultaneous detection of fluorescence and chemiluminescence in real-time to discriminate between $ONOO^-$ and H_2O_2 generation (Shuhendler et al., 2014), making this a particular promising approach for the detection of specific ROS in HSCs. However, further studies are needed to determine whether these probes can be used in HSCs.

Conclusions

In view of the data demonstrating that an anaerobic metabolic character is related to the maintenance of stemness, methods to accurately measure the levels of ROS and RNS in stem cells are of paramount importance. These highly reactive molecules have regulatory roles in energy metabolism that might impact on the HSC fate. Current evidence indicates that variations in the levels of ROS and RNS affect the balance between HSC self-renewal and commitment. As discussed above for commonly used techniques for the measurement of reactive species, there are clear imprecisions in the currently used approaches that must be overcome in order to help us to better understand how ROS and RNS impact on HSC biology. We suggest here that, by combining new advances in the detection of specific ROS and RNS with protocols that allow to achieve a higher enrichment of HSCs, it will be possible to confirm and elucidate any of functional roles of ROS and RNS in stem cell maintenance.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

M.V.L. and Z.I. designed and wrote the manuscript.

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