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Research Paper

Redox environment in stem and differentiated cells: A quantitative approach

O.G. Lyublinskaya[a,*], Ju.S. Ivanova[a,b], N.A. Pugovkina[a], I.V. Kozhukharova[a], Z.V. Kovaleva[a], A.N. Shatrova[a], N.D. Aksenov[a], V.V. Zenin[a], Yu.A. Kaulin[a], I.A. Gamaley[a], N.N. Nikolsky[a]

[a] Department of Intracellular Signaling and Transport, Institute of Cytology, Russian Academy of Sciences, Tikhoretsky pr. 4, St. Petersburg 194064, Russia
[b] Department of Medical Physics, Institute of Physics, Nanotechnology and Telecommunications, Peter the Great St. Petersburg Polytechnic University, Polytechnicheskaya st. 29, St. Petersburg 195251, Russia

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ABSTRACT

Stem cells are believed to maintain a specific intracellular redox status through a combination of enhanced removal capacity and limited production of ROS. In the present study, we challenge this assumption by developing a quantitative approach for the analysis of the pro- and antioxidant ability of human embryonic stem cells in comparison with their differentiated descendants, as well as adult stem and non-stem cells. Our measurements showed that embryonic stem cells are characterized by low ROS level, low rate of extracellular hydrogen peroxide removal and low threshold for peroxide-induced cytotoxicity. However, biochemical normalization of these parameters to cell volume/protein leads to matching of normalized values in stem and differentiated cells and shows that tested in the present study cells (human embryonic stem cells and their fibroblast-like progenies, adult mesenchymal stem cells, lymphocytes, HeLa) maintain similar intracellular redox status. Based on these observations, we propose to use ROS concentration averaged over the cell volume instead of ROS level as a measure of intracellular redox balance. We show that attempts to use ROS level for comparative analysis of redox status of morphologically different cells could lead to false conclusions. Methods for the assessment of ROS concentration based on flow cytometry analysis with the use of H_2DCFDA dye and HyPer, genetically encoded probe for hydrogen peroxide, are discussed.

1. Introduction

Quantitative Redox Biology (QRB) is a new trend in life sciences that emphasizes the transition of modern biology from observational to analytical science [1]. QRB approach is aimed at collecting quantitative information on the content and oxidation state of redox-active compounds as well as thermodynamic/kinetic parameters of their reactions in living cells and bodies [2–5]. Quantitative estimates are necessary to understand the basic principles of redox interactions and redox regulation within fundamental cellular systems such as metabolome [6], proteome [7], lipidome [8], and genome [9,10]. In this paper, we use QRB approach for the comparative analysis of the redox status of stem and differentiated cells, and discuss possible quantification strategy for the proper assessment of the ROS content in different cultured cells.

The change in the basal ROS level in cells is an important indicator of their functional state. There is a large body of data dedicated to the redox state of the cell and the oxidative stress that leads to cell damage [11–13]. Nevertheless, the precise method for determining the amount of ROS in a cell is yet to be discovered. There is a direct colorimetric method for quantitation of ROS progenitor molecule (superoxide anion radical) that estimates amount of ROS based on oxidation/reduction of a specific substrate. However, the method is not totally specific [14] and is suitable only for immune cells [15,16], which generate hundreds-fold more ROS molecules than any other type of cells. Besides, there are a number of fluorescence assays for evaluating ROS levels in a wide range of non-immune living cells, which are based on usage of different fluorogenic substrates. A vast variety of such probes is reviewed in details in [17–19]. The present study mostly concentrates on the usage of cell-permeable non-fluorescent (but fluorescein-containing) probe 2', 7'-dichlorodihydrofluorescein diacetate (H_2DCFDA). Although the concept of this method goes back a long way [20,21], the method remains commonly used up to the present day [22]. In a modern version of the assay, ROS levels are detected by measuring fluorescence of the oxidized form of the probe using mostly flow cytometry. In spite of many disadvantages and limitations of this method [14,19,23,24], H_2DCFDA-based assay is the most widespread.

Abbreviations: ROS, reactive oxygen species; QRB, quantitative redox biology; H_2DCFDA, 2', 7'-dichlorodihydrofluorescein diacetate; ESCs, embryonic stem cells; EB, embryoid bodies; difESCs, differentiated ESC progenies; eMSCs, endometrial mesenchymal stem cells; PHA, phytohemagglutinin; DTT, dithiothreitol; ROI, region of interest; HRP, horseradish peroxidase

* Corresponding author.
E-mail address: o.lyublinskaya@mail.ru (O.G. Lyublinskaya).

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It is H_2DCFDA that has been used for the probing of ROS in stem cells in a number of published works [25–28].

Stem cell studies have shown that embryonic stem cells, as well as adult stem cells, possess a very low ability to oxidize H_2DCFDA probe in comparison with their differentiated progeny [25,27,28]. Furthermore, it has been shown that the expression level of many antioxidant genes in stem cells is higher than that in differentiated cells [29–31]. Based on these data, the concept of a specific redox balance in stem cells was formulated. Redox environment inside stem cells is believed to differ from that of differentiated descendant cells due to combination of enhanced removal capacity and limited production of ROS [25,32]. It makes sense because these cells exist in specific physiological niches of an organism in oxygen-depleted environment and differ from their differentiated counterparts in the energy metabolism, favoring the glycolytic pathway as a source of energy production [33,34]. However, taking into account the significant difference between metabolic, physiological and morphological characteristics of stem and differentiated cells, comprehensive comparative analysis of redox homeostasis requires combination of qualitative and quantitative approaches.

The goal of this work is to develop a quantitative approach for the analysis of the pro- and antioxidant ability of embryonic stem cells in comparison with their differentiated descendants, as well as adult stem and non-stem cells. To solve this problem, we tried to elucidate the reasons for the low ability of stem cells to oxidize ROS-sensitive probes based on fluorescein and compared the results of H_2DCFDA-based analysis with the data obtained with HyPer, genetically encoded sensor of hydrogen peroxide [35,36]. We also assessed the rate of elimination of hydrogen peroxide in the micromolar concentration range by adding hydrogen peroxide to the culture medium of stem and differentiated cells and compared the thresholds of H_2O_2-induced cytotoxicity.

2. Materials and methods

2.1. Cell cultures

2.1.1. Embryonic stem cells (ESCs)

Two lines of human embryonic stem cells C910 and C612 have been exploited throughout the study [37]. Both cell lines showed similar characteristics in the experiments. Cells exhibit typical for ESCs morphology, express pluripotency markers, have diploid karyotype and capacity for the differentiation into the three germ layer cells [37]. ESCs were routinely cultured in mTeSR1 medium (Stem Cell Technology) in Petri dishes on feeder layers of mitotically inactivated human endometrial mesenchymal stem cells [38] and passaged mechanically. For the experiments, cells were grown upon the Matrigel matrix (BD Biosciences), or, if indicated, upon different feeder layers: mitotically inactivated mouse embryonic fibroblasts, mitotically inactivated human endometrial mesenchymal stem cells, or feeder layer composed of ESC differentiated progenies.

2.1.2. Differentiated ESC progenies (difESCs)

ESCs were spontaneously differentiated through embryoid bodies (EB) formation. ESC colonies were cut in pieces and plated into non-adhesive plastic dishes in the DMEM/F12 medium supplemented with 10% fetal bovine serum, 1% non-essential amino acids, 0.1 mM β-mercaptoethanol, antibiotics (ICN). Within one week, cystic EB were formed. After that, EB were transferred into the tissue culture dishes and cultivated in DMEM/F12 medium with 10% fetal bovine serum, 1% L-glutamine, and 1% penicillin-streptomycin. Spontaneously differentiated cells derived from the attached EBs were subcultured once a week at the split ratio 1:3. After 2–3 passages, the culture was composed of morphologically homogenous fibroblast-like cells which lost the pluripotency markers and expressed the mesenchymal marker vimentin.

2.1.3. Endometrial mesenchymal stem cells (eMSCs)

Human endometrial mesenchymal stem cells were isolated from a desquamated endometrium of menstrual blood from healthy donors [38] and demonstrated properties typical for the mesenchymal stem cell cultures [39–41]. eMSCs were cultivated in DMEM/F12 medium supplemented with 10% fetal bovine serum, 1% L-glutamine, and 1% penicillin-streptomycin. Cells from the 3rd to 25th passages were subcultured twice a week at the split ratio 1:3.

2.1.4. HeLa cells

Cells were obtained from the Russian Cell Culture Collection (Institute of Cytology, St. Petersburg, Russia) and cultivated in DMEM/F12 medium supplemented with 10% fetal bovine serum, 1% L-glutamine, and 1% penicillin-streptomycin at 37 °C, 5% CO_2. Cells were subcultured twice a week at the split ratio 1:5.

2.1.5. U118 glioma cell line

Cells were received from American Type Culture Collection (USA). Cells were cultivated in 199 medium supplemented with 10% fetal bovine serum at 5% CO_2, 37 °C.

2.1.6. Lymphocytes

Human lymphocytes were kindly gifted by Dr. Irina I. Marakhova (Institute of Cytology, St. Petersburg, Russia). Cells were isolated from fresh venous blood of healthy donors according to the procedure described in [42] and suspended in RPMI-1640 medium supplemented with 2 mM glutamine and 5% heat-inactivated human serum (AB IV Rh +). To activate quiescent lymphocytes for proliferation, at the next day after isolation, the cell suspension (> 85% CD3+ cells) was adjusted to the concentration of $1.5 \cdot 10^6$ cells/ml, was added with phytohemagglutinin (PHA-M, Sigma, USA) at the final concentration of 10 μg/ml and incubated at 37 °C in a humidified chamber with 5% CO_2 for 48 h.

2.2. Immunofluorescence

Cells grown on coverslips were fixed with 4% formalin in phosphate-buffered saline (PBS), permeabilized with 0.1% Triton X-100, incubated with 1% bovine serum albumin for 40 min to block a non-specific binding, treated with the primary antibodies for 1 h, washed with PBS/0.1% Tween-20, treated with secondary antibodies for 1 h, washed with PBS/0.1% Tween-20 and counterstained with 1 μg/ml DAPI. The coverslips were mounted in 2% propylgallate and visualized under an Axiovert 200 M microscope (Carl Zeiss, Germany) equipped with a Leica DFC 420 C camera (Germany). Primary antibodies used in this study are mouse monoclonal anti-OCT-3/4 (C-10) in dilution 1:100 (Santa Cruz Biotechnology, USA), anti-TRA-1–81 in dilution 1:50 (Millipore, USA), anti-Vimentin in dilution 1:50 (Sigma, USA) and polyclonal rabbit anti-SOX2 in dilution 1:100 (Abcam, USA).

2.3. ROS Measurements

2.3.1. Flow cytometry

For the detection of intracellular ROS level we used ROS-sensitive probe 2',7'-dichlorodihydrofluorescein diacetate (H_2DCFDA, Invitrogen, D-399). H_2DCFDA was dissolved in DMSO to obtain a 10 mM stock solutions and further diluted before use. Adherent cells (ESCs, difESCs, eMSCs, HeLa, U118) were incubated with 5 μM staining solution in PBS in the dark for 30 min at 37 °C, then harvested with 0.05% trypsin-EDTA solution, suspended in a fresh medium, and immediately analyzed with flow cytometer (Epics XL, Beckman Coulter, USA; 488 nm laser). Lymphocytes, both control and PHA-activated, were resuspended in PBS, incubated with 5 μM of H_2DCFDA in the dark for 30 min at 37 °C, and immediately analyzed. Along with the H_2DCFDA probe, if indicated, we used ROS-insensitive modification of the fluorescein dye 2',7'-dichlorofluorescein diacetate (DCFDA). The staining procedure was the same as for the H_2DCFDA.

2.3.2. Confocal microscopy

For the simultaneous visualization of the oxidized H_2DCFDA fluorescence in ESCs and difESCs, ESCs were grown on the coverslips upon the difESCs used as a feeder layer, and so both cell types were treated with H_2DCFDA and analyzed simultaneously. Coverslips with cells were placed in the 5 μM H_2DCFDA staining solution in PBS for 60 min at 37 °C in the dark, then washed and imaged with a confocal laser-scanning microscope Leica TCS SL equipped with an argon laser. Signal quantification was performed using ImageJ software (US National Institutes of Health). In brief, a region of interest (ROI) was manually drawn along the cell membrane in each cell. Next, the mean value of fluorescence intensity per the cell unit area [43] (denoted as DCF/area) and the mean integrated density of fluorescence collected from one cell (denoted as DCF/cell) were analyzed within the ROI.

2.4. Normalization of ROS level to cell volume/protein

In a separate series of experiments, for the ROS quantification per the cell volume or protein content, after the H_2DCFDA treatment, cells were harvested with 0.05% trypsin-EDTA solution and resuspended in the fresh medium. One half of the suspension was subjected to the ROS measurements, while the other one was subjected to the cell volume measurements, or, alternatively, to the cell counting and Bradford protein assay [44]. For the cell protein content determination, measured total protein was normalized to the number of cells. Cell volume was estimated either from measurements of the mean diameter of suspended cells in the counting chamber, or from the direct cell volume measurements using Scepter™ 2.0 Cell Counter (Merck Millipore, USA).

2.5. HyPer-based assay

ESC, difESC and eMSC cells were transfected with HyPer expression vector using commercially available plasmid pHyPer-cyto (Evrogen, Russia) and FuGene 6 (Promega, USA) transfection reagent [45]. Transfection mixtures were prepared according to the manufacturer's instructions at a 7:2 ratio of FuGene to DNA. 36–48 h after transfection, cells were harvested with 0.05% trypsin-EDTA solution, suspended in a fresh medium, incubated in suspension for 30 min in standard growth conditions (37 °C, 5%CO_2) for adaptation to a new environment, and analyzed with flow cytometer (CytoFLEX, Beckman Coulter, USA; 405/488 nm laser). Before the analysis, suspension was split into 3 probes: the first one was analyzed immediately, the second one was analyzed after 5 min incubation with 1 mM of H_2O_2, while the rest part of suspension was incubated for 10 min with 30 mM of dithiothreitol (DTT) and then analyzed. During the analysis, cells were gated for HyPer expression (see Supplement, Fig. 2S), and within this gate the mean ratio of EX488/FL530 and EX405/FL520 signals (denoted here and after as 488/405 ratio) was determined. Intracellular peroxide concentration was assessed using HyPer-index (H), which was quantified in %% as follows:

$$H = (R_{cells} - R_{DTT})/(R_{H2O2} - R_{DTT}),$$

where R_{cells} is 488/405 ratio measured in HyPer$^+$ intact cells, while R_{DTT} and R_{H2O2} are ratio values measured in the same cells after incubation with DTT and H_2O_2 correspondingly. In all tested cells, along with the HyPer-index, intracellular pH was flow cytometrically controlled using BCECF AM dye (2',7'-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein, acetoxymethyl ester, Molecular Probes, USA) applied in accordance with the manufacture's instructions.

2.6. Extracellular H_2O_2 removal assay

Rate of extracellular H_2O_2 scavenging by cells was measured using the Amplex® Red Hydrogen Peroxide/Peroxidase Assay Kit (Invitrogen, USA) that contains Amplex Red reagent and horseradish peroxidase (HRP). Cell cultures were grown for 48 h in the 35 mm Petri dishes, washed with 2 ml of warm (37 °C) PBS, and H_2O_2 was then added to the dish at a final concentration in the range of 5–25 μM. Just after that, every 3 min, 10 μL aliquots were removed from the dish maintained at 37 °C, mixed with the Amplex® Red reagent/HRP working solution, and residual H_2O_2 concentration was quantified by measuring the absorbance of the solution with the Multiskan FC microplate photometer (Thermo Scientific, USA) after 30 min incubation. After the measurements, cells were harvested with 0.05% trypsin-EDTA solution, counted and subjected to the Bradford assay [44] for the total protein quantification. The rate of H_2O_2 removal by cells was approximated [46] by a first-order rate law,

$$d[H2O2]/dt = -k[H2O2],$$

where k is the first-order rate constant measured in s^{-1} and derived from the slope of the [H2O2] drop in the logarithmic scale. The rate constant quantified per one cell will be,

$$k_{cell} = k/(cell\ L^{-1}),$$

where ($cell\ L^{-1}$) is the number of cells divided by the total volume of PBS buffer in the well, in liters. The rate constant quantified per gram of the cell protein will be,

$$k_{prot} = k/(Protein\ L^{-1}),$$

where ($Protein\ L^{-1}$) is the total cell protein divided by the total volume of PBS buffer in the well, in liters.

2.7. Cell viability assay

To compare the H_2O_2 toxic effect on ESCs and difESCs, cells were grown for 48 h, then placed to the DMEM/F12 medium supplemented with ITC solution (Gibco, UK), and exposed to H_2O_2 at various concentrations for 24 h. Cell viability was estimated by flow cytometry using propidium iodide staining. Cells were harvested with 0.05% trypsin-EDTA solution, suspended in a fresh medium, treated with 50 μg/ml propidium iodide and analyzed with flow cytometer (Epics XL, Beckman Coulter, USA; 488 nm laser). H_2O_2 doses were quantified either on the mole-per-cell basis [47] by the normalization of the H_2O_2 molar quantity to the number of cells in the dish at the moment of H_2O_2 addition [48], or in the mole-per-protein units by the normalization of the H_2O_2 molar quantity to the total cell protein in the dish at the moment of H_2O_2 addition.

2.8. Statistical analysis

All experiments were repeated at least 3 times. Data are presented as means ± SD, when indicated. Statistical significance was evaluated by t-test, and $P < 0.05$ was considered to be significant. The degree of linear correlation was estimated by Pearson product-moment correlation coefficient. All flow cytometry histograms and microscopy images shown throughout the paper correspond to the most representative experiments.

3. Results

3.1. Characterization of ESCs and differentiated progeny cells

ESCs exploited throughout the study exhibited normal ESC morphology and expressed key pluripotency markers Oct3/4, Sox2 and TRA 1–81 (Fig. 1A-D). Differentiated ESC progeny (difESCs) were derived from ESCs by spontaneous differentiation according to the method described in [49] via the embryoid body formation (Fig. 1E). DifESC cultures were composed of morphologically homogenous fibroblast-like cells expressing the mesenchymal marker vimentin (Fig. 1F, G).

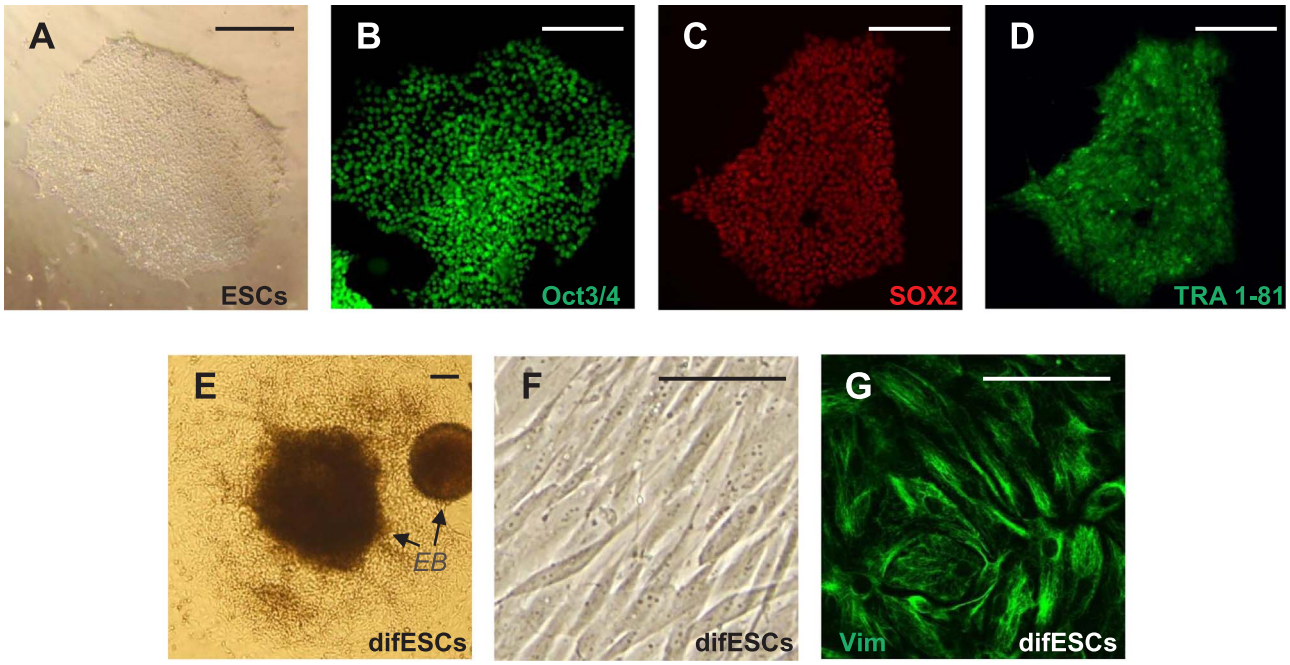

Fig. 1. Characterization of embryonic stem cells (ESCs) and their fibroblast-like differentiated progenies (difESCs). (A) Typical morphology of ESC clone. (B-D) Immunofluorescence analysis of ESC clones for the expression of pluripotency markers Oct3/4, SOX2 and TRA 1–81. (E) Generation of difESCs from ESCs: cell morphology at day 2 after the embryoid bodies plating. (F) Morphology of difESCs at passage 5. (G) Immunofluorescence analysis of difESCs for the expression of mesenchymal marker vimentin. Scale bar, 100 μm. Abbreviations: EB, embryoid bodies; Vim, vimentin.

3.2. Basal ROS level in ESCs and difESCs

Analysis of H_2DCFDA probe oxidation by means of flow cytometry technique showed that the basal level of ROS in difESCs is about 6–7 times larger than that in ESCs (Fig. 2A, left panel). This observation is in accord with previously published data obtained with the same fluorescent probe in the studies of stem and differentiated cells [26–28]. Our experiments revealed that the signal from the oxidized dye in ESCs was sensitive to the variation of the intracellular ROS level caused by pro- and antioxidants, was stable during at least one hour after cell staining and did not depend on the substrate for growing ESCs (matrigel, mouse embryonic fibroblast feeder layer, or human endometrial mesenchymal stem cell feeder layer) (see Supplement, Fig. 1S).

To find out whether the weak fluorescence of the oxidized H_2DCFDA in ESCs suggests a highly specific intracellular redox environment in stem cells, we used 2',7'-dichlorofluorescein diacetate (DCFDA), ROS-insensitive modification of the dichlorofluorescein dye. Both H_2DCFDA and DCFDA probes are non-fluorescent in their initial form but they undergo multistep conversion inside the cell (Fig. 2B) that results in the formation of fluorescent product dichlorofluorescein (DCF). The only difference between these two probes is that the conversion of H_2DCFDA involves oxidation. Therefore, fluorescence of the H_2DCFDA-treated cells depends on the intracellular ROS level, in contrast to fluorescence associated with the DCFDA probe. Surprisingly, flow cytometry analysis showed that the difference between fluorescence levels of ESCs and difESCs loaded with H_2DCFDA (Fig. 2A, left panel) is close to the difference between the signals from DCFDA-treated cells (Fig. 2A, right panel). The latter indicates a ROS-independent reason for low fluorescent signal of oxidized H_2DCFDA in ESCs.

Next, we employed confocal laser scanning microscopy for the comparative visualization of the fluorescence from ESCs and difESCs treated with H_2DCFDA. ESCs were grown on the coverslips upon the difESCs used as a feeder layer so that both cell types can be treated with H_2DCFDA and analyzed simultaneously. Visually estimated intensity of fluorescence from ESCs and difESCs (Fig. 2C) was about the same. Analysis of the microscopy images using the ImageJ software confirms these observations. Measurements of the mean value of fluorescence intensity within the cells (i.e. signal collected per unit area of the cell) show that the difference in the mean values (Fig. 2D, DCF/area) for ESCs and DifESCs is negligible. At the same time, comparison of integrated density of the cell fluorescence (i.e. signal collected from the whole area of the cell) shows the 8-fold difference between ESCs and difESCs in favor of difESC (Fig. 2D, DCF/cell), that is very close to the results obtained with flow cytometry. This means that concentration of the oxidized H_2DCFDA is about the same in ESCs and DifESCs, whereas the amount of oxidized dye per cell is different mainly due to the different cell sizes. Fig. 2E shows histograms of the cell size distribution for ESCs and difESCs, obtained by measuring the diameter of the suspended cells in the counting chamber. Mean values of ESCs and difESCs diameters differ nearly twice (Table 1). Accordingly, the difference in the volumes of two cell types turned out to be much more obvious. Our measurements of the mean cell volume with the usage of the Scepter™ Cell Counter revealed about the 6-fold difference in the volumes of ESCs and DifESCs (Table 1). In order to find out whether the difference in ESCs' and difESCs' cell sizes is the main reason for the difference in the ROS levels revealed by flow cytometry assay of H_2DCFDA-loaded ESCs and difESCs (Fig. 2D, mean DCF), we assessed the oxidized dye concentration in these cells, using the normalization of the cytometric signal from the H_2DCFDA-treated cells to the measured cell volume, or, alternatively, to the cell protein content (Table 1) determined in the same probes (Fig. 2D, DCF/volume and DCF/protein). Similarly to the microscopy-based estimations, concentration of the oxidized H_2DCFDA dye in ESCs probed by flow cytometry occurred to be very close to that in difESCs. Thus, our experiments showed that the ability of the cell's unit volume to oxidize H_2DCFDA in ESCs and difESCs is quite similar, which challenges the hypothesis about the highly specific redox environment in stem cells.

3.3. Basal ROS level in ESCs in comparison with other cell cultures

Next, using flow cytometry, we compared the ROS level in ESCs with that in other human stem and non-stem cell cultures. We exploited mesenchymal stem cells derived from the endometrium (eMSCs) (as an

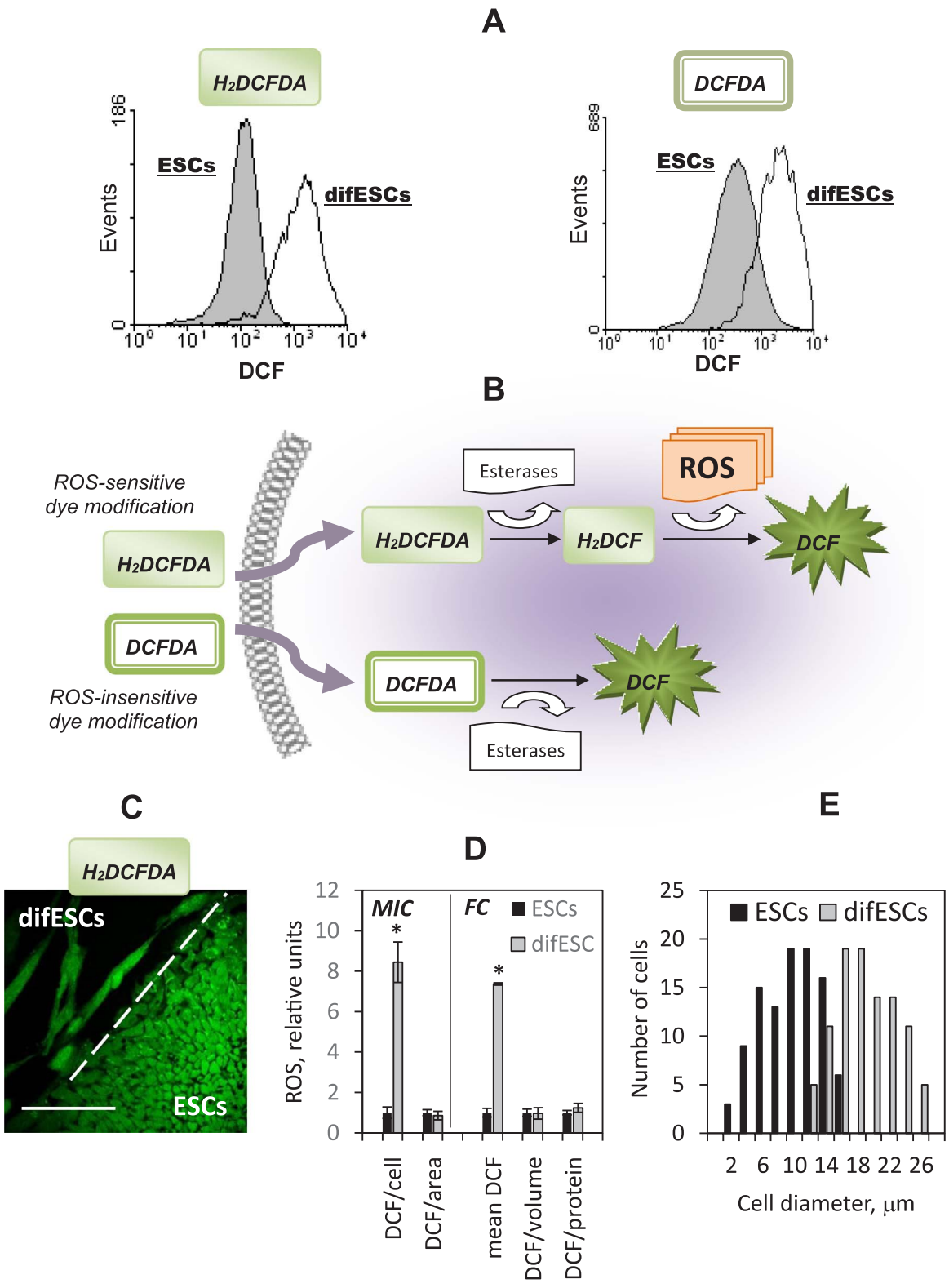

Fig. 2. ROS level measured by flow cytometry using H_2DCFDA dye in embryonic stem cells (ESCs) and their differentiated progenies (difESCs): effect of the cell size. (A) Flow cytometry histograms of ESCs and difESCs treated with the ROS-sensitive H_2DCFDA (left panel) and the ROS-insensitive DCFDA (right panel) fluorescein-based dyes. (B) Scheme demonstrating intracellular multistep conversion of the ROS-sensitive (H_2DCFDA) and ROS-insensitive (DCFDA) dyes. (C) Confocal microscopy image of H_2DCFDA-treated ESCs grown upon difESC feeder layer. Scale bar, 100 μm. (D) Oxidized H_2DCFDA fluorescence signal quantified per cell (DCF/cell and mean DCF) or per cell area/volume/protein (DCF/area, DCF/volume, DCF/protein,) in ESCs and difESCs. Signal was measured either by flow cytometry (right panel), or by processing of the confocal microscopy images of the cells (left panel). Data are normalized to the ESC fluorescence and presented as mean ± SD (n = 3, * $P < 0.01$). (E) Cell size distribution for ESCs and difESCs obtained by measuring the diameter of the suspended cells in the counting chamber. Abbreviations: H_2DCFDA, 2',7'-dichlorodihydrofluorescein diacetate; DCFDA, 2',7'-dichlorofluorescein diacetate; DCF, 2',7'-dichlorodihydrofluorescein, FC, flow cytometry; MIC, microscopy.

Table 1
Physical parameters of tested cells: embryonic stem cells (ESCs), their differentiated progeny cells (difESCs) and endometrial mesenchymal stem cells (eMSCs).

Parameter	Cells		
	ESCs	**difESCs**	**eMSCs pas.7 − 9**
Cell diameter, μm	10 ± 3 (N ≥ 100)	19 ± 4 (N ≥ 100)	21 ± 4 (N ≥ 100)
Cell volume, pL	0.58 ± 0.02 (N = 3)	3.6 ± 0.2 (N = 3)	4.3 ± 0.4 (N = 3)
Cell protein content, ng	0.070 ± 0.006 (N = 3)	0.34 ± 0.06 (N = 3)	0.50 ± 0.04 (N = 3)

example of adult stem cells), HeLa cells (as an example of stable cell line) and lymphocytes (as an example of primary cell culture). Throughout this series of experiments we analyzed cells at different passages by estimating in each cell sample both the oxidized H_2DCFDA fluorescence signal (as a measure of ROS level) and the cell protein content (as a measure of cell volume). In some cell lines, such as ESCs and HeLa, the ROS level and cell protein content were independent of the cell passage, whereas in others (for example eMSCs and difESCs), both parameters significantly increased with passage number (see Fig. 3A). Taken together, our measurements revealed strong correlation between the oxidized H_2DCFDA fluorescence signal and cell protein content in all tested cells with a Pearson coefficient equal to 0.73 (Fig. 3B). When normalized to the cell protein, signals of oxidized H_2DCFDA fluorescence in different cells were found to be very close to each other (Fig. 3C). This means that a given intracellular volume of all tested cells, including ESCs and DifESCs, shows a similar ability to oxidize H_2DCFDA dye in normal conditions, and that the main reason for the discrepancy in the measured ROS level in these cells is a different cell size. It is interesting to note that the latter is true not only for different cell cultures, but also for eMSC cells probed for ROS at different passages (Fig. 3D). In the literature on the subject, the increase in the ROS level with passaging is often attributed to a shift in the intracellular redox balance in aged cells and considered to be a

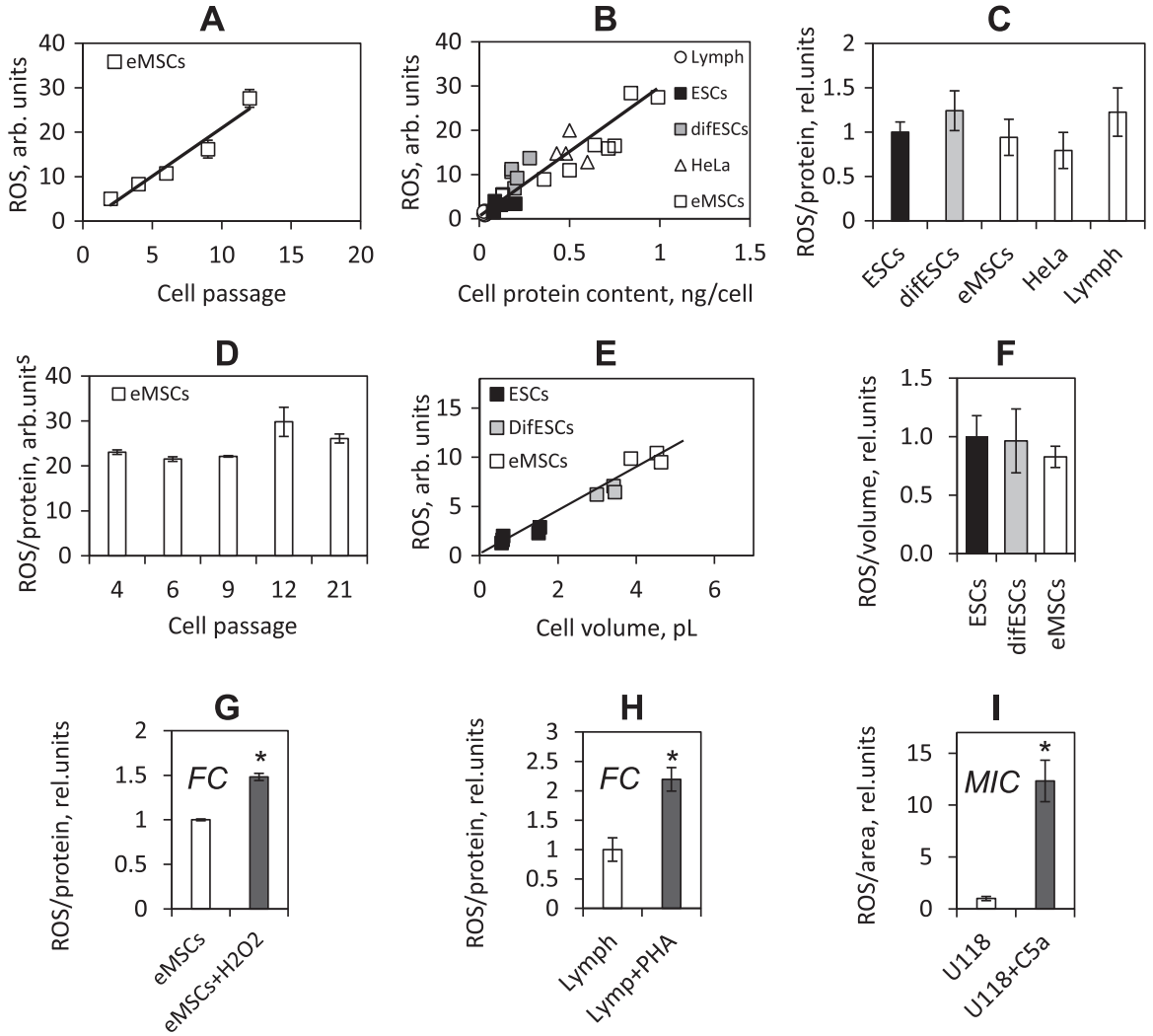

Fig. 3. Cultivated human stem and non-stem cells vary in their basal ROS level, but have similar ROS concentration. ROS are detected by flow cytometry using H_2DCFDA dye. (A) ROS level in adult mesenchymal stem cells derived from endometrium (eMSCs) vs. cell passage number. (B, C) ROS in embryonic stem cells (ESCs), their differentiated progenies (DifESCs), lymphocytes (Lymph), eMSCs and HeLa cells: positive correlation between the ROS level and the cell protein content (B), ROS concentration quantified by normalization of the ROS level to the cell protein (C). (D) ROS concentration in eMSCs vs. cell passage number. (E, F) Positive correlation between the ROS level and the cell volume (E) as well as ROS concentration quantified by normalization of the ROS level to the cell volume (F) in ESCs, DifESCs and eMSCs (passage 7). (G-I) ROS concentration in control and stimulated cells: (G) eMSCs treated with 100 μM of H_2O_2; (H) lymphocytes stimulated with phytohemagglutinin (PHA) for 48 h; (I) astrocytic tumor cells U118 activated with anaphylatoxin C5a. ROS level is measured either by flow cytometry (G, H), or by microscopy (I), and normalized either to the cell protein content (G, H) or to the cell area (I) for ROS concentration assessment. Data are presented as mean ± SD (n ≥ 3), * $P < 0.01$. Abbreviations: ESCs, embryonic stem cells; difESCs, differentiated embryonic stem cells; eMSCs, endometrial mesenchymal stem cells; HeLa, HeLa cells; Lymph, lymphocytes; Lymph + PHA, lymphocytes stimulated with phytohemagglutinin; U118, astrocytic tumor cell line; U118 + C5a, U118 astrocytes treated with complement component - anaphylatoxin C5a; FC, flow cytometry; MIC, microscopy.

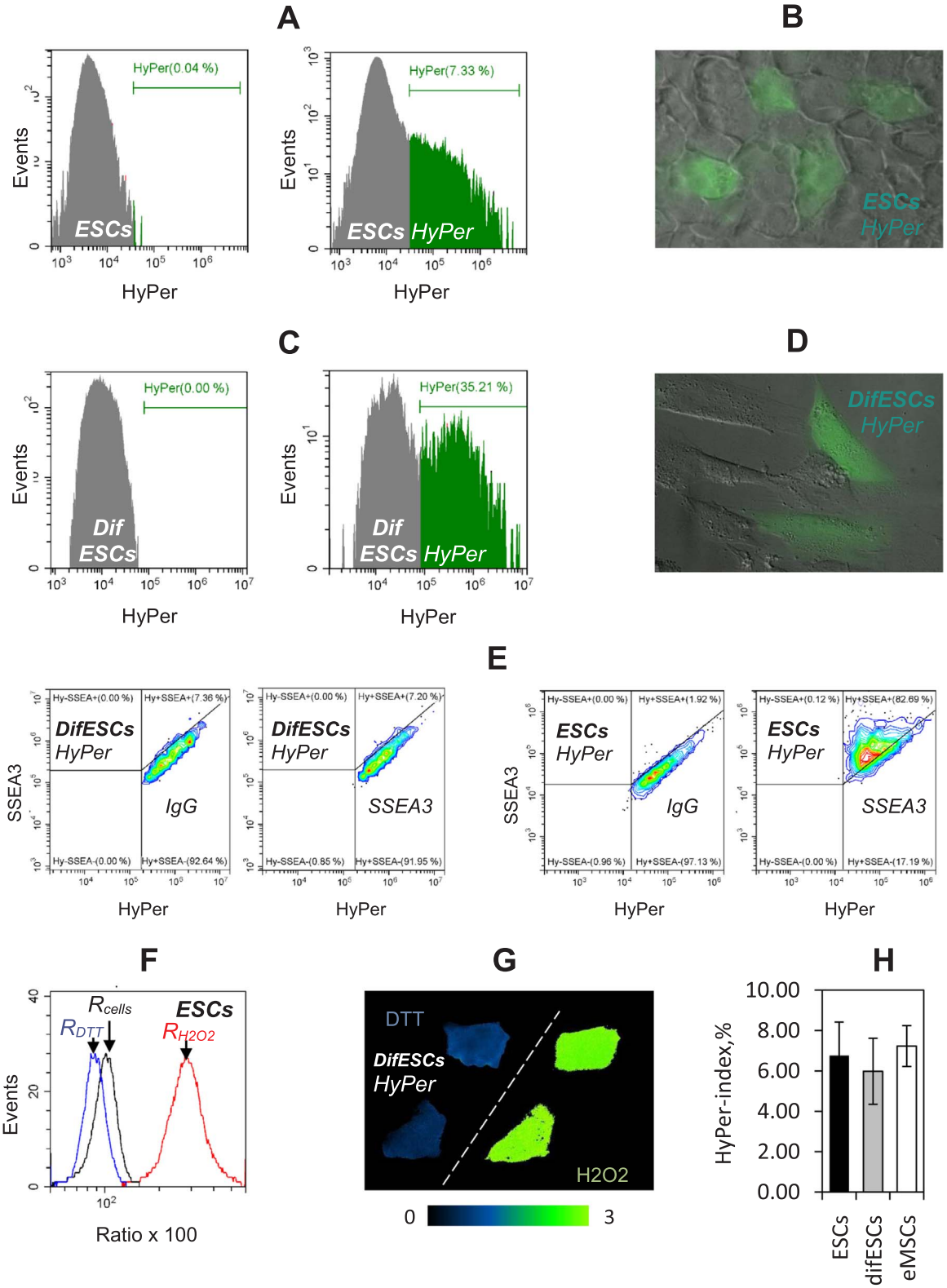

Fig. 4. Flow cytometry HyPer-based assay of embryonic stem cells (ESCs), their differentiated progenies (DifESCs), and adult mesenchymal stem cells derived from endometrium (eMSCs, passage 8): intracellular H_2O_2 concentration is similar. (A, C) Histograms of ESCs (A, right panel) and DifESCs (C, right panel) transfected with HyPer in comparison to the control cells treated with the transfection reagent FuGene (left panels). (B, D) Microscopy images of ESC colony (B) and DifESC culture (D) expressing HyPer. (E) Test for the pluripotency marker SSEA3 expression by cell populations expressing HyPer: left panels – DifESC, right panels – ESCs. Cells were probed with SSEA3 as well as isotype control (IgG) antibodies. (F) Histogram displaying 488/405 ratio (multiplied by 10^2) in intact HyPer$^+$ ESCs, as well as HyPer$^+$ ESCs treated with 1 mM of H_2O_2 or 30 mM of DTT. (G) Ratiometric confocal image of DifESCs expressing HyPer treated with 1 mM of H_2O_2 (right) or 30 mM of DTT (left). (H) HyPer-index calculated for ESCs, DifESCs and eMSCs as $(R_{cells}–R_{DTT})/(R_{H2O2}–R_{DTT})$. Data are presented as mean ± SD ($n \geq 3$). In (E, F, H), cells were gated for HyPer expression using forward scatter versus HyPer fluorescence plot according to the gating strategy described in the Supplement (Fig. 2S). Abbreviations: DTT, dithiothreitol; R_{cells}, 488/405 ratio in intact HyPer$^+$ cells; R_{DTT}, 488/405 ratio in HyPer$^+$ cells treated with DTT; R_{H2O2}, 488/405 ratio in HyPer$^+$ cells treated with H_2O_2.

hallmark of the cell senescence [50] during in-vitro mesenchymal stem cell expansion [51–53]. Our experiments proved that this effect could be explained by an increase in the cell volume of adult stem cells rather than by changes in the intracellular redox balance during cell passaging.

Thus, our study showed that flow cytometric measurements of ROS level per se occur to be uninformative, as this type of assay never allows comparing morphologically different cells. Normalization of ROS level to the cell protein content that in fact yields a parameter which is close to ROS concentration is more suitable for comparative cell studies. Along with the cell protein, it is possible to use other normalization approaches. In a separate series of experiments, for the estimation of ROS concentration in ESCs, DifESCs and eMSCs, we used normalization of the ROS level, measured with flow cytometry using H_2DCFDA, to the cell volume (measured using Scepter™ Cell Counter). In tested cells we found correlation between the ROS level and the cell volume (Fig. 3E), and accordingly, ROS concentration, evaluated with this method, occurred to be similar in these cells (Fig. 3F).

To check the ability of different cells to oxidize H_2DCFDA dye in the conditions of elevated ROS we used a number of stimuli, such as hydrogen peroxide, anaphylatoxin C5a, or phytohemagglutinin (PHA) and compared the ROS concentration assessed with different methods in control and stimulated cells (Fig. 3G-I). By means of flow cytometry, using the normalization of the ROS level to the cell protein content, we estimated ROS concentration in control and H_2O_2-treated eMSCs, as well as in unstimulated and PHA-stimulated lymphocytes. In the microscopy experiments, evaluating fluorescence intensity normalized to the cell area, we assessed ROS concentration in human glioma U118 cells, both control and stimulated with 1 nM anaphylatoxin C5a. In all cases, our measurements confirmed the redox status changing in the result of cell stimulation.

Summarizing briefly the results of the current sub-section, until we consider H_2DCFDA oxidation as a measure of endogenous ROS, basal ROS concentration in all tested cell lines, including ESCs and difESCs, is quite similar but can be disturbed by cell stimulations.

3.4. ROS status in ESCs and difESCs probed with the genetically encoded sensor HyPer

It is now well-established [14,19,23,24] that oxidation of H_2DCFDA is catalyst- and context-dependent. It is influenced by the variety of intracellular parameters such as acidity, activity of esterases that provide the probe trapping inside the cells, availability of catalysts and so on. In this context, the best way to validate our H_2DCFDA-based measurements is to use an alternative methodology for the assessment of the ROS balance in ESCs and difESCs. For this purpose, we chose HyPer, genetically encoded fluorescent probe for hydrogen peroxide [35]. This probe allows monitoring the intracellular H_2O_2 levels with a high degree of sensitivity and specificity and is widely used for the ratiometric imaging of the cells. Microscopic techniques provide an opportunity to visualize the H_2O_2 fluxes in intact cells [54,55], and thus is a method of choice for single cell analysis. In contrast, flow cytometry is suitable for large massive data collection, so we use multi-color flow cytometry ratiometric analysis for the comparative study of the ROS balance in ESCs and difESCs. Ratiometric method is a perfect way to eliminate analysis ambiguity arising from the difference of stem and differentiated cells in their morphology.

Fig. 4 shows the histograms of the transfected cells, as well as the corresponding microscopy images of ESCs and difESCs expressing HyPer. Data were collected 36 h after transfection, when the fraction of HyPer+ cells was about 5–8% in the case of ESCs (Fig. 4A, B) and 15–30% in the case of difESCs (Fig. 4C, D). In ESC cultures, HyPer+ cells were positive for pluripotency marker SSEA3, in contrast to DifESC cells (Fig. 4E). In spite of the huge diversity in the fluorescence intensity of transfected cells within each sample, the ratio of EX488/FL530 and EX405/FL520 signals, denoted here and after as 488/405 ratio, was almost the same, and the corresponding histograms, which depict ratio within the fraction of HyPer+ cells, had small dispersions (Fig. 4F and Fig. 2S in the Supplement). Cell treatments with H_2O_2 and dithiothreitol (DTT) shifted the ROS balance (see, for example, the ratiometric microscopy images in Fig. 4G), and we used total reduction and total oxidation of HyPer with 30 mM DTT and 1 mM H_2O_2 correspondingly for the calibration of our measurements. We designated the shift of 488/405 ratio from the totally reduced state (defined as 0%) towards totally oxidized state (defined as 100%) as HyPer-index *H* quantified in %% [55]. HyPer-indexes derived from the measurements of 488/405 ratio in HyPer+ ESCs, difESCs and eMSCs occurred to be the same (about 6 ± 1%, see Fig. 4H), that confirms the results of H_2DCFDA-based analysis and supports the hypothesis about the similar ROS status of tested cells.

3.5. Rate of the external H_2O_2 removal in ESCs and difESCs

Next, we evaluated the antioxidant defense potential in ESCs, their differentiated progenies and adult stem cells by comparing the rates of the external hydrogen peroxide elimination. In these experiments, H_2O_2 was added to the cells at the concentration over a range from 5 to25 μM. Photometric measurements of extracellular H_2O_2 concentration ($[H_2O_2]$) in the cell medium aliquots, removed from the cell dish and assayed with Aplex Red reagent/HRP solution, showed that extracellular $[H_2O_2]$ began to drop immediately after H_2O_2 addition due to its fast utilization by cells. Kinetics of the H_2O_2 quantity decay revealed an exponential dependence which can be well approximated as first order kinetics [46]. For both ESCs and difESCs, the rate of H_2O_2 removal was depended on the number of cells in the dish (Fig. 5A, B). The slope of the line presented in Fig. 5A–C indicates the rate at which external H_2O_2 is removed by cells, i.e. the rate constant which is independent on the initial H_2O_2 quantity in the tested H_2O_2 concentration range. ESCs showed very low H_2O_2 elimination rate constant per one cell ($k_{cell} = 3.0 \pm 0.5 \times 10^{-12}\ s^{-1}\ cell^{-1}\ L$) in comparison to both difESCs and eMSCs (Table 2 and Fig. 5C, D). Surprisingly, the rate of the H_2O_2 removal by ESCs calculated per gram of the cell protein ($k_{prot} = 3.7 \pm 0.6 \times 10^{-2}\ s^{-1}\ g^{-1}\ L$) turned out to be very close to that in difESCs. It was even higher than that in eMSCs (Table 2 and Fig. 5E). Thus, small cell size turned out to be the major reason for a low capacity of ESCs to remove extracellular H_2O_2. Perhaps this is the reason why ESCs are extremely sensitive to H_2O_2 cytotoxicity. Fig. 5F, G demonstrates viability of ESCs and difESCs after 24-h H_2O_2 bolus exposure, estimated as a number of viable cells normalized to the control values. Viability, assessed by flow cytometry analysis using propidium iodide staining, is presented as a function of H_2O_2 dose. The latter was quantified either on the mole-per-cell basis (Fig. 5F) [47] by the normalization of the H_2O_2 molar quantity to the number of cells in the dish at the moment of H_2O_2 addition [48], or in the mole-per-protein units (Fig. 5G) by the normalization of the H_2O_2 molar quantity to the total cell protein. Cytotoxic threshold was assessed as H_2O_2 dose which resulted in 50% cell viability (LD50). Table 2 shows that cytotoxic threshold estimated on the mole-per-cell basis ($LD50_{cell}$) is 1.0 ± 0.3 pmole/cell for ESCs and 11 ± 1 pmole/cell for difESCs. However, the difference between the same parameters calculated on the per-protein basis ($LD50_{prot}$) is much less (12 ± 4 and 20 ± 2 mmole/g for ESCs for difESCs, respectively).

4. Discussion

QRB approach to the study of cell redox homeostasis began to develop just recently [56]. One of the QRB tasks is to implement informative metrics for the quantification of basic redox parameters. Here, we discuss the appropriate metrics for the comparative assay of ROS related parameters in cultured cells and use these metrics as well as previously developed QRB approaches to study ROS environment inside embryonic stem cells and their differentiated progeny cells.

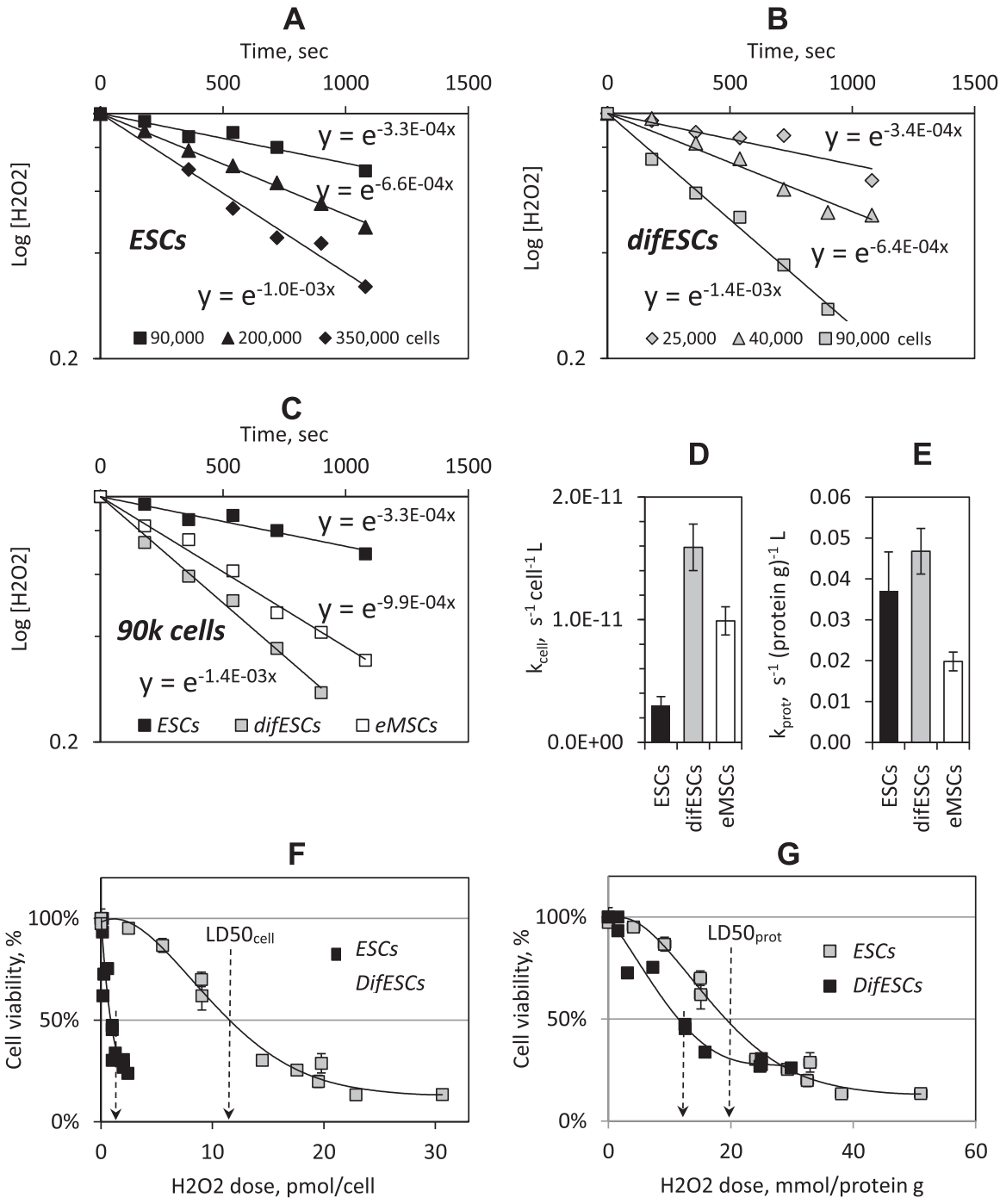

Fig. 5. Impact of H_2O_2 bolus addition on embryonic stem cells (ESCs), their differentiated progenies (difESCs) and adult mesenchymal stem cells derived from endometrium (eMSCs, passage 7–9): effect of the cell size. (A-C) Photometric measurements of $[H_2O_2]$ decay in the cell medium approximated by a simple first-order rate equations. Medium aliquots were removed from the cell dishes after addition of 5 μM of H_2O_2 and assayed with Aplex Red/HRP reagent. (A) H_2O_2 was added to the cell dishes containing different number of ESC colonies. Cells were counted after measurements, and cell number is indicated in the graph. (B) H_2O_2 was added to the cell dishes containing different number of difESCs. (C) H_2O_2 was added in parallel to 3 cell dishes with equal number (90,000) of cells (ESCs, DifESCs, or eMSCs). (D, E) First-order rate constant for the H_2O_2 removal by ESCs, difESCs and eMSCs quantified on the per-cell (D), or per-protein (E) basis. Data are presented as mean ± SD (n = 3). (F, G) ESC and difESC viability measured as a percent of propidium iodide-negative cells by flow cytometry after 24 h-incubation with different H_2O_2 doses quantified in the mole-per-cell (F), or mole-per-protein (G) units. Data are normalized to the control values and presented as mean ± SD (n = 3). Abbreviations: k_{cell} and k_{prot}, first-order rate constants for the external H_2O_2 removal quantified on the per-cell, or per-protein basis, correspondingly; $LD50_{cell}$ and $LD50_{prot}$, cytotoxic thresholds assessed as H_2O_2 dose which resulted in 50% cell death and estimated either in mole-per-cell, or mole-per-protein units, correspondingly.

Our experiments confirmed that the measurement of ROS levels with flow cytometry by using H_2DCFDA probe in stem and differentiated cells depends not only on the amount of intracellular ROS and other biochemical parameters which affect the oxidation of the probe [14,19,23,24], but also on the cell size (Fig. 2). The diameter of ESCs in suspension turned out to be twice as small as that of difESCs and therefore ESCs are several times smaller in volume. Fluorescence signal of H_2DCFDA detected by flow cytometry reflects the oxidized dye content in the cell and is proportional to cell volume. Thus, low level of ROS detected in ESCs by using H_2DCFDA is caused mainly by small geometric size of these cells. Flow cytometry measurements with signal normalization to the cell volume or cell protein, showed that the intracellular concentration of oxidized H_2DCFDA was about the same in the ESC and difESC. These results challenge the widespread hypothesis

Table 2
Hydrogen peroxide elimination ($[H_2O_2] = 5$ μM) and toxicity. Cell cultures: embryonic stem cells (ESCs), their differentiated progeny cells (difESCs) and endometrial mesenchymal stem cells (eMSCs).

	Cells		
Parameter	**ESCs**	**difESCs**	**eMSCs**
k_{cell}, s^{-1} $cell^{-1}$ L	$3.0 \pm 0.5 \times 10^{-12}$(N = 5)	$16 \pm 2 \times 10^{-12}$ (N = 4)	$10 \pm 1 \times 10^{-12}$ (N = 4)
k_{prot}, s^{-1} g^{-1} L	$3.7 \pm 0.6 \times 10^{-2}$ (N = 5)	$4.7 \pm 0.5 \times 10^{-2}$ (N = 4)	$2.0 \pm 0.2 \times 10^{-2}$ (N = 4)
$LD50_{cell}$, pmole/cell	1.0 ± 0.3 (N = 3)	11 ± 1 (N = 2)	
$LD50_{prot}$, mmole/protein g	12 ± 4 (N = 3)	20 ± 2 (N = 2)	

Notations: k_{cell} and k_{prot}, first-order rate constants for the external H_2O_2 removal quantified on the per-cell, or per-protein basis, correspondingly; $LD50_{cell}$ and $LD50_{prot}$, cytotoxic thresholds assessed as H_2O_2 dose which resulted in 50% cell death and estimated either in mole-per-cell, or mole-per-protein units, correspondingly.

that stem cells have a highly specific redox status. Furthermore, our H_2DCFDA-based assay showed that intracellular concentration of oxidized H_2DCFDA in ESCs was similar to that in other primary- and non-primary human cell cultures: mesenchymal stem cells, adult lymphocytes, and HeLa cells (Fig. 3). Our measurements proved that the intracellular unit volume in all tested cells had essentially the same ability to oxidize the probe that indicates the same intracellular ROS concentration. These findings allowed us to advance the provocative hypothesis that intracellular ROS concentration in normal cells appears to be some kind of physiological constant. We suggest that interplay between the pro- and antioxidant systems raises and lowers ROS concentration in cells, depending on their current physiological status, but these oscillations occur near some regular value, which is almost the same in all normal cells. Of course, ROS concentration is a nominal parameter composed from contributions of different chemical substances and averaged over the whole cell volume. Local concentration of the redox-active compounds may substantially differ from the value averaged over the whole cell [54], and we suggest using the concept of ROS concentration solely for the rough estimation of the cellular redox status in comparative cell studies.

Unquestionably, various pathologic processes as well as a cell stimulation can disturb regular ROS concentration. Our comparison of oxidized H_2DCFDA concentration in unstimulated cells and in cells treated with PHA, H_2O_2, or anaphylatoxin C5a demonstrated enhanced ability of stimulated cells to oxidize H_2DCFDA (Fig. 3G–I). The latter indicates a change in the cellular redox status after the stimulations. At the same time, we did not find changes in the intracellular ROS concentration in the cultures of mesenchymal stem cells after long-term cell passaging. It turned out that when the H_2DCFDA fluorescence signal was increased by almost an order of magnitude in the result of long-term cell cultivation (Fig. 3A), the concentration of oxidized probe in cells at different passages was nearly the same (Fig. 3D). Thus, in our experiments, variation in ROS level mainly reflected changes in the morphology rather than homeostasis of aged cells. In cultured mesenchymal stem cells, rise in the level of ROS is being often interpreted as a shift in ROS homeostasis and thus is considered as a marker for the cell replicative senescence [51–53]. Our comparative study of aged and young cells showed that ROS concentrations rather than ROS levels should be compared and analyzed.

The term "ROS level", which is currently used to characterize cell redox status, is itself highly controversial. ROS level accounts for many redox-active molecules such as hydrogen peroxide, superoxide radical, hydroxyl radical, etc., and application of this term masks our inability to differentiate between them. In addition, it turns out that measurements of ROS levels by using H_2DCFDA are often differently interpreted depending on the detection method used. Here, we show that microscopy-based assays [43], which specify ROI within the cell and evaluate the mean fluorescence intensity within this ROI, imply that the measured fluorescence signal is proportional to the intracellular concentration of the oxidized dye. In other words, they reflect ROS concentration in the cell. The same parameter can be evaluated by using microplate reader [43] provided that measured signal is normalized to the cell protein. On the other hand, flow cytometrical signal is proportional to the amount of oxidized dye in the cell, which, in turn, indicates intracellular ROS content that is dependent on the cell volume. However, in all cases, the measured signal is often referred to as "ROS level". This dual interpretation is irrelevant as long as we use H_2DCFDA-based measurements for the characterization of the redox status of cells of the same size (for example, when comparing cells before and after some treatment), but it acquires a very different meaning in the comparative analysis of cells of different sizes (such as old/young, resting/proliferating, transformed/non-transformed and differentiated/stem cells). We believe that normalization of ROS level measured by flow cytometry to the amount of cell protein or cell volume should become a standard procedure for the comparative analysis of redox homeostasis in different cell cultures, which makes the term "ROS concentration" more acceptable. In fact, developed for the comparative analysis of protein expression techniques use the similar approach, i.e. normalization of intracellular protein content to the loading control or to the total protein.

To analyze ROS concentration by flow cytometry using H_2DCFDA dye it is sufficient to normalize the fluorescence signal to the cell volume (using, for example, a cell counter with the function of cell volume measurement) or any other parameter reflecting cell volume, such as cell protein content. Besides, it is possible to use other normalization approaches that have been previously elaborated for the comparative analysis of ROS homeostasis in cells, such as normalization of oxidized H_2DCFDA fluorescence to the mean cell autofluorescence [57] or rate of oxygen consumption [58]. For the normalization of fluorescence of mitochondria-targeted ROS-sensitive probes, it is possible to assess cell mitochondrial mass by using 10-N-Nonyl acridine orange (NAO) [59]. Normalization of the quantity of intracellular ROS to cell protein/volume (ROS concentration) is a conventional biochemical approach, while expressing ROS amount normalized per cell (ROS level) can be considered as a biophysical approach. Similar normalization approaches has been used to determine xenobiotics effectiveness [47]. Both metrics yield ROS quantification in arbitrary units, but biochemical approach is more suitable for the comparative studies.

Another elegant way to estimate in arbitrary units intracellular ROS concentration, and even more specifically hydrogen peroxide concentration, is to use ratiometric analysis based on fluorescence properties of HyPer, genetically encoded fluorescent probe for hydrogen peroxide [35,36,45]. HyPer-index measured in our study by flow cytometry allows comparing H_2O_2 concentration in cells of various morphology and different physiological status. It is important to note, that flow cytometry measurements enable high-throughput estimation of H_2O_2 concentration in different subpopulations of cells in testing samples, as well as simultaneous multiparametric analysis. Further elaboration of precise H_2O_2 calibration procedure might provide quantitative estimations of H_2O_2 intracellular concentration in HyPer-based experiments. The only limitation of this method is a necessity of pH control [36], which can be achieved by using various fluorescent probes, such as BCECF AM (2',7'-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein, acetoxymethyl ester). HyPer-indexes derived from our analysis of

HyPer-expressing ESCs, difESCs and eMSCs occurred to be the same (Fig. 4) that confirms the results of H_2DCFDA-based analysis and supports the hypothesis about the similar ROS status of tested cells.

Intracellular redox status is determined not only by the cell ability to produce ROS, but also by the ability to eliminate ROS through the antioxidant system [60,61]. One of the ways to evaluate the capacity of the cell antioxidant system is to measure the rate of removal of extracellular hydrogen peroxide after H_2O_2 bolus addition [62]. Studies implementing the QRB approach have shown that at micromolar concentrations of extracellular H_2O_2 the removal rate can be approximated by a rate equation that is first-order in the concentration of H_2O_2 and cell density, and that the rate constants quantified on the per cell basis can be used to compare the antioxidant capacity of cells [46]. Similarly, it has been suggested to apply the per cell basis as an informative dosing metrics in the comparative studies of cells' susceptibility to the cytotoxic action of H_2O_2 and various xenobiotics [47,63–65]. It has been shown that for a correct assessment of cytotoxicity it is preferably to use the concept of dose of a substance, expressed in units of moles per cell [47]. This approach seems to be mandatory if the substance is consumed during its metabolization by cells (as in the case of H_2O_2).

Using previously elaborated QRB approaches, we demonstrate here that embryonic stem cells have low capacity to remove extracellular H_2O_2 applied at micromolar concentrations in comparison with their differentiated descendants and adult stem cells (Fig. 5A-C). Being quantified on the per cell basis, rate constant of H_2O_2 removal (Fig. 5D) and threshold dose for H_2O_2-induced cytotoxicity (Fig. 5F) were found to be extremely low in ESCs. However, the normalization of the rate constants of peroxide elimination (Fig. 5E), as well as thresholds for cytotoxicity of peroxide (Fig. 5G), on the cell protein content led to similar values for all tested cells. This means that small geometrical dimensions of embryonic stem cells result in a low level of intracellular antioxidants that defines a low rate of H_2O_2 elimination of foreign peroxide by cell and high sensitivity of cells to the cytotoxic effects.

Thus, we show that when basic redox parameters of embryonic stem cells, such as ROS content, capacity to remove hydrogen peroxide or tolerance to H_2O_2 oxidative action, are quantified per the cell, they do not reflect the characteristics of the unique stem-cell-like redox homeostasis. Biochemical normalization of the main redox parameters to cell volume/protein, in contrast, shows that cultured stem and differentiated cells have the similar redox status. It should be noted however that this conclusion is related to cultured cells that live in the laboratory environment. Introduction to cell culture inevitably results in cell adaptation to the growth under laboratory conditions and we can hardy generalize our observations to cells in the living body.

Finally, it is worth noting that our findings are in accord with the recent data on metabolic profiling of MEL-2 embryonic stem cells [66]. Turner and colleagues showed that highly active glycolysis in MEL-2 ESCs is supported by the oxidative phosphorylation within the functional mitochondria, which utilize carbon sources, such as glutamine, to maximize ATP production. Presented in [66] quantitative analysis of metabolic fluxes revealed the conventional Warburg effect typical for highly proliferative cell cultures instead of a unique stem-cell-like oxidative metabolism in ESCs.

5. Conclusions

In conclusion, we carried out a comparative analysis of the redox status of human embryonic stem cells, their differentiated descendants, as well as adult stem and non-stem cells. This analysis refines the commonly accepted ideas about highly specific redox environment in stem cells. We show that in order to asses ROS status of morphologically different cells it is necessary to evaluate ROS concentration rather than ROS level in the cell. To achieve this in H_2DCFDA-based microscopy or flow cytometry assay it is sufficient to normalize cell fluorescence signal to cell area or cell volume, correspondingly. Other normalization approaches, such as normalization to cell protein, are also possible. Attempts to use the concept of ROS level, which implies measuring an integrated fluorescence intensity of redox-sensitive probe for comparative assessment of redox status of different cells, could lead to false conclusions. A highly informative way to compare ROS status of different cells is to estimate intracellular H_2O_2 concentration with the use of HyPer, genetically encoded probe for H_2O_2, by measuring HyPer-index with flow cytometer.

Declaration of interest

The authors declare that they have no competing interests.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.redox.2017.04.016.

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