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Hypoxia/Hypercapnia-Induced Adaptation Maintains Functional Capacity of Cord Blood Stem and Progenitor Cells at 4°C

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We analyzed the effect of exposure to hypoxic/hypercapnic (HH) gas mixture (5% O_2/9% CO_2) on the maintenance of functional cord blood $CD34^+$ hematopoietic stem and progenitor cells in severe hypothermia (4°C) employing the physiological and proteomic approaches. Ten-day exposure to HH maintained the Day 0 (D-0) level of hematopoietic stem cells as detected in vivo on the basis of hematopoietic repopulation of immunodeficient mice—short-term scid repopulating cells (SRC). Conversely, in the atmospheric air (20% O_2/0.05% CO_2), usual condition used for cell storage at 4°C, stem cell activity was significantly decreased. Also, HH doubled the survival of $CD34^+$ cells and committed progenitors (CFCs) with respect to the atmospheric air (60% vs. 30%, respectively). Improved cell maintenance in HH was associated with higher proportion of aldehyde dehydrogenase (ALDH) positive cells. Cell-protective effects are associated with an improved maintenance of the plasma and mitochondrial membrane potential and with a conversion to the glycolytic energetic state. We also showed that HH decreased apoptosis, despite a sustained ROS production and a drop of ATP amount per viable cell. The proteomic study revealed that the global protein content was better preserved in HH. This analysis identified: (i) proteins sensitive or insensitive to hypothermia irrespective of the gas phase, and (ii) proteins related to the HH cell-protective effect. Among them are some protein families known to be implicated in the prolonged survival of hibernating animals in hypothermia. These findings suggest a way to optimize short-term cell conservation without freezing.

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Storage in severe hypothermia (4°C), for more than few days triggers cellular injuries (Louis et al., 2012). Primary cause of damage comes from the cold-induced mismatch between ATP supply and demand leading to cellular energetic deficit. Consequently, the failure of ion-motive ATPases is followed by an impairment of ionic balance. This leads to membrane depolarization and uncontrolled influx of Ca^{2+} through voltage-gated Ca^{2+} channels resulting in the activation of Ca^{2+}-dependent hydrolases and in turn provokes further membrane depolarization, uncontrolled cell swelling and ultimately, cell necrosis (Hochachka, 1986). These events are potentiated with behavior of plasma membrane lipids in cold which undergo phase transition interfering with membrane function and leads to ions leakage (Quinn, 1985). Recent studies showed that cold-induced cell injuries are associated with an increase of available cellular labile iron pool, initiating the formation of reactive oxygen species (ROS). The increase in ROS levels is usually followed by a massive lipid peroxidation and alteration of mitochondrial function which can lead to cell necrosis in hypothermia or apoptosis upon rewarming (Rauen et al., 1999; Kerkweg et al., 2003; Rauen and de Groot, 2004).

Hibernating animals are capable of surviving at near freezing temperatures for a long time without injuries. Although underlying mechanisms are not fully understood, it is known

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that this cold tolerance is accomplished by a highly regulated metabolic depression (Guppy et al., 1994) which anticipates the decrease in body temperature (Heldmaier et al., 2004). The metabolic depression is manifested by the attenuation of the ATP consuming processes (i.e., transcription, translation, synthesis of proteins, cells division, differentiation, suppression of ion movement across the membrane—"channel arrest") and by increased efficacy of the ATP production (Hochachka, 1986; Storey and Storey, 2007). These events are carried out by the selective gene expression which enables protective mechanisms to maintain stable ATP turnover and cellular homeostasis in hypothermia (Van Breukelen and Martin, 2002; Carey et al., 2003; Storey and Storey, 2004).

It was suggested that hypoxia/hypercapnia triggered hypothermia-protective metabolic depression during entrance into hibernation (Wasser et al., 1990; Hand and Hardewig, 1996; Boutilier, 2001). Joint action of hypothermia, hypoxia, and hypercapnia induces development of respiratory acidosis and the artificial hibernation in non-hibernating animals (Ignat'ev et al., 1989; Mel'nychuk et al., 1995) which enables their revival after cooling to body temperature at $0-1°C$ (Andjus and Smith, 1955).

On the basis of our "oxygen stem cell paradigm" (Ivanovic, 2009) we assumed that primitive hematopoietic cells exhibit "hibernation-like" intrinsic properties. We hypothesized that the same hypothermia protective mechanisms could be induced by a hypoxic/hypercapnic gas mixture in heterogeneous ($CD34^+$) cell population (Ivanovic, 2010).

Recently, we showed that incubation in 3% O_2/6% CO_2 atmosphere improved maintenance in hypothermia of $CD34^+$ cells mobilized in peripheral blood (Jeanne et al., 2009). Relatively long persistence of stem cells in situ postmortem can be also considered in the context of hypothermia combined with hypoxia, hypercapnia, and ischemia (Latil et al., 2012). Furthermore, the resistance to hypothermia of "good mobilizers" $CD34^+$ cells is impaired in parallel with a higher degree of the proliferation and metabolic activation of these cells with respect to their counterparts from "poor mobilizers" which are less active and more resistant (Ivanovic et al., 2010). Together, these findings indicate towards the hypo-metabolic state which seems to confer the resistance to hypothermia (Boutilier, 2001).

The present study shows that the beneficial effect of the hypoxia/hypercapnia on survival of stem and progenitor cells inside cord blood (CB) $CD34^+$ cell population is associated with better maintenance of a part of the proteome despite sustained ROS formation.

Materials and Methods

$CD34^+$ cells processing

Purification. CB samples delivered (with the mother's approval) to the Cell Therapy Unit of the French Blood Institute, Bordeaux, rejected for banking, were used in experiments. $CD34^+$ cells from umbilical CB were isolated by immunomagnetic technique (Miltenyi Biotec, Paris, France).

Conservation. $CD34^+$ cells (5×10^4/ml) were plated in Stemα S3 (Stem Alpha SA, Saint Genis, l'Argentière, France), incubated in air (20% O_2/0.05% CO_2) or in the hypoxic (1% and 5% O_2)/hypercapnic atmosphere (2.5% and 9% CO_2) for 10 days at 4°C. All the tests were performed with purified (D-0) and preserved $CD34^+$ cells after 10 days at 4°C (D-10). Desired gas mixtures were obtained in O_2 and CO_2 controllers equipped culture chamber (PRO-OX and PRO-CO_2, Biospherix, Lacona, NY). For the estimation of $CD34^+$ cell maintenance in acidified media, pH was decreased by the addition of HCl.

Expansion. Harvested D-10 $CD34^+$ were cultivated (10^4/ml) for 7 days in the HP01 medium (Macopharma, Tourcoing, France) supplemented with SCF, G-CSF, and MGDF (Amgen, Neuilly-sur Seine, France; all at 100 ng/ml) at 37°C in atmosphere with 20% O_2/5% CO_2 (Incubator Igo 150 Cell Life, Jouan, St. Herblain, France) (Ivanovic et al., 2006).

Stem/progenitors cell assays

CFC assay. The committed progenitors CFCs ([CFU-GM] plus [BFU-E]) were assayed as previously described (Ivanovic et al., 2004). Briefly, D-0 and D-10 $CD34^+$ cells (250 cells/ml and 600 cells/ml, respectively) were seeded in methylcellulose Stem Alpha-1D (Stem Alpha, Stem Alpha Saint Genis l'Argentière, France). After 14 days incubation, CFCs were counted.

Aldehyde dehydrogenase (ALDH) activity. ALDH labeling was performed using Aldefluor reagent (ALDF) (Stem Cell Technologies, Grenoble, France) according to the manufacturer's instructions. Briefly, activated ALDF substrate was added to 10^5 $CD34^+$ cells suspended in 100 μl Aldefluor assay buffer and incubated for 30 min at 37°C (Storms et al., 2005). As a negative control, an aliquot of ALDF-stained cells was incubated with ALDH inhibitor diethylaminobenzaldehyde (DEAB).

Scid repopulating cells (SRC) assay. The animal experiments were performed in compliance with the French regulation (License No: 3306002). 10^3 D-0 or D-10 $CD34^+$ cells were injected into 6- to 10- week-old NOD-scid IL2rγ^{null} (NSG) mice (animal-keeping facility, University Bordeaux Segalen, France) conditioned by intraperitoneal injection of Busulfan 25 mg/kg (Busilvex, Pierre Fabre, Boulogne, France) as described previously (Robert-Richard et al., 2006). Positive control (mice injected with 2×10^4 D-0 $CD34^+$ cells) and negative control (non-injected mice) were included. After 6 weeks, the animals were euthanized, their femora were isolated and the bone marrow (BM) was flushed with 1 ml RPMI 1640 (Eurobio, Courtabeuf, France), supplemented with HSA (0.4%) (Vialebex, LFB-biomedicament, Courtabeuf, France). Cells were washed with PBS (Lonza, Verviers, Belgium), EDTA 5 mM (Sigma Aldrich, St. Louis, MO) and human serum albumin (0.4%), stained with anti-human antibodies: CD45-FITC and CD19-PE for 20 min at 4°C in the dark and analyzed on a FACSCalibur (BD Bioscience). In order to evaluate human CFCs in the murine BM, femoral suspensions were seeded in methylcellulose (Stem alpha-1; Stem Alpha, SA) supplemented with 10% human plasma AB, 25 ng/ml rHu IL-3 (PeproTech, Rocky Hill, NJ), 25 ng/ml rHu GM-CSF (R&D Systems Europe, Lille, France), 50 ng/ml rHu SCF and 3 U/ml rHu erythropoietin (Tebu-Bio, Le Perray, France). After 14 days incubation human CFCs were counted. SRC assay as performed here detects a heterogeneous population of hematopoietic multipotent progenitors and stem cells. However, with respect to subpopulation which this assay detects predominantly, it is generally considered as measure of short-term reconstitution activity (Ivanovic et al., 2011).

Metabolic assays

ATP levels were quantified by ATP bioluminescent somatic cell assay kit (Sigma) in accordance with the manufacturer's recommendations. A total of 10^5 cells /well were used in 96-well plate.

"*Cellular oxidative stress*" was determined using 2′,7′-dichlorodihydrofluorescein-diacetate (H_2DCF-DA) (Molecular Probes, Invitrogen, Eugene, OR) (Jakubowski and Bartosz, 2000). 10^5 $CD34^+$ cells were treated with 10 μM H_2DCF-DA at 37°C for 30 min.

Superoxide anion ($O^{\cdot-}_2$) spin trapping was done by electron paramagnetic resonance (EPR). $CD34^+$ cells (1.5×10^5) were incubated in Stemα S3 containing 500 μM 1-hydroxy-3-methoxycarbonyl-2,2,5,5-tetramethylpyrrolidin (CMH) (Noxygen), 25 μM deferoxamine (Sigma) and 5 μM *N*,*N*-diethyldithiocarbamate (Sigma) in Stem alpha 3 at 37°C for 45 min. Reaction was stopped by freezing the sample in liquid nitrogen.

Samples were then analyzed by EPR spectrometry on a tabletop x-band spectrometer miniscope (MS200, Magnettech, Berlin, Germany). Spectra of the oxidized product of CMH were recorded at 77uK using a Dewar flask. Acquisition parameters were as followed: Bo Field: 33416 ± 150 G, microwave power: 10 dB, amplitude modulation: 5 G, sweep time: 60 sec, gain: 300 and 3 scans (Billaud et al., 2009). Signals were quantified by measuring the total amplitude after correction of baseline.

Measurement of hydrogen peroxide (H_2O_2) released from the CD34$^+$ cells using Amplex red. CD34$^+$ cells (2×10^5) in Krebs–Ringer-phosphate glucose buffer were added in the pre-warmed reaction mixture containing 50 μM of Amplex Red reagent and 0.5 U/ml Horseradish peroxidase (Molecular Probes). After 1 h incubation at 37°C, fluorescence was measured (Varioskan, Thermo Scientific) at 590 nm (Mohanty et al., 1997). H_2O_2 values released from the preserved CD34$^+$ cells were determined using H_2O_2 standard curve.

Mitochondrial membrane potential was assessed using tetramethylrhodamine methyl ester perchlorate (TMRM) (Molecular Probes) (Scaduto and Grotyohann, 1999). 10^5 CD34$^+$ cells were treated with TMRM (25 nM) at 37°C for 20 min. To exclude dead cells, 7-amino-actinomycin (7-AAD) was added (BD Bioscience).

Plasma membrane potential was measured with bis-(1,3-dimethylbarbituric acid) trimethine-oxonol (DiBAC4(3)) (Wilson and Chused, 1985). 10^5 CD34$^+$ cells were treated with DiBAC4(3) (150 nM) at 37°C for 30 min. To exclude non-viable cells, propidium iodide (PI) (10 μg/ml) was added (Sigma).

Intracellular pH (pH_i) measurement was assessed with carboxyseminaphtorhodafluor-1-acetoxymethyl ester (SNARF-1) (Whitaker et al., 1991). 10^5 CD34$^+$ cells were loaded with 10 μM SNARF-1 at 37°C for 30 min. Excitation was at 488 nm, while fluorescence emission was monitored at two wavelengths 580 and 640 nm. Fluorescence response was calibrated using nigericin (10 μM) (all from Molecular Probes) in the presence of potassium enriched buffer with defined pH (pH 6–7.4) (Owen, 1992). pH_i was determined according the equation:

$$pH_i = pKa - \log\left[\frac{R - R_b}{R_a - R} \times F_b(\lambda 2)/Fa(\lambda 2)\right]$$

R: ratio Fλ1/Fλ2 of fluorescence intensities (F) measured at two wavelengths λ1 (580 nm) and λ2 (640 nm); the subscripts a and b: limiting values at the acidic and basic endpoints of the titration; pKa: negative logarithm of the SNARF-1 dissociation constant, K_a.

Apoptosis was determined with Annexin V-FITC kit (Beckman Coulter, Roissy, France) following the manufacturer's protocol. Briefly, 10^5 CD34$^+$ cells were labeled with Annexin V-FITC solution (AnnV) and propidium iodide (PI) (10 μg/ml) for 15 min at 4°C.

Bioenergetic analysis of preserved cells

Oxygen consumption rate (OCR) as an indicator of mitochondrial respiration (Varum et al., 2011) and extracellular acidification rate (ECAR) as indicator of glycolytic activity (Wu et al., 2007) were determined simultaneously using XF24 Analyzer. D-0 and D-10 CD34$^+$ cells preserved in air or hypoxia/hypercapnia (5% O_2/9% CO_2) were resuspended in bicarbonate-free DMEM (SeaHorse Bioscience) only (glycolytic activity estimation) or supplemented with 1 mM pyruvate and 10 mM glucose (mitochondrial activity estimation). 2×10^5 cells/well were plated in the Cell-TAK (BD Bioscience) (7.9 μg/cm^2) coated 24-well XF V7 plate. The cells were allowed to become attached for 30 min in a 37°C non-CO_2 incubator. It should be stressed that the cells from all conditions were transferred to atmospheric air at 37°C to perform the measurements. Mitochondrial respiration was monitored in the basal state and after sequential injections of the mitochondrial modulators oligomycin (1 μM), 2,4 DNP (100 μM), and rotenone (1 μM) (mitochondrial stress test) (Varum et al., 2011). Glycolytic activity was estimated in the basal state and after sequential injections of 10 mM glucose, oligomycin (1 μM) and 2-deoxy-glucose (100 mM) (glycolysis stress test) (Wu et al., 2007). All chemicals were purchased from Sigma.

Proteomic analysis

D-0 and D-10 CD34$^+$ cells preserved (10^6) in air, hypoxia/hypercapnia (5% O_2/9% CO_2), hypercapnia (20% O_2/9% CO_2), or hypoxia (5% O_2/0% CO_2) were analyzed with a proteomic approach. Adequate numbers of cells were obtained by pooling CD34$^+$ cells isolated from 10 to 20 CB units. Samples were lysed and proteins reduced, alkylated then digested with trypsin as previously described (Negroni et al., 2007). After Sep-Pak C18 for peptides cleaning, each sample was labeled with a specific iTRAQ reagent (named 113–117 for the corresponding reporter ions) according to the manufacturer's recommendations (ITRAQ 8 Plex kit; AB SCIEX, les Ulis, France). iTRAQ Reagents were: 113 for D-0 CD34$^+$ cells, 114–117 for D-10 CD34$^+$ cells preserved in air, hypoxia/hypercapnia, hypercapnia, or hypoxia only, respectively. The labeled samples were mixed then purified on SCX chromatography and analyzed with a nano LC system coupled with a LTQ Orbitrap XL (Thermo Fisher Scientific, Courtaboeuf, France) as previously described (Przybylski et al., 2010). Peptides identification (false discovery rate <1%) and iTRAQ quantitation was performed with Proteome Discoverer 1.2 (Thermo Fisher Scientific). iTRAQ ratio was expressed with 113 as a reference. A protein was considered differently expressed in respect to the reference when at least three peptides were quantified and a fold change (greater than 1.2 or smaller than 0.8) was statistically significantly different (Unwin et al., 2006). Protein function was attributed according to the protein accession number using UniPROTKB/Swiss-Prot database.

Statistical analysis

The Wilcoxon paired comparison test was used to examine the significance of difference between experimental conditions, while the Mann–Whitney test was used for in vivo experiments with mice and proteomic analysis ($P < 0.05$ was considered statistically significant). Proportions were compared by χ^2 test.

Results

Hypoxia/hypercapnia ensures conservation of CD34$^+$ cells, committed progenitors (CFCs), cell expansion potential, and aldehyde dehydrogenase (ALDH) activity in hypothermia

Incubation in hypoxia/hypercapnia, for 10 days at 4°C significantly increases the maintenance of CD34$^+$ cells (77 ± 14%) in all tested gas mixtures (1% O_2/2.5% CO_2, 1% O_2/9% CO_2, 5% O_2/2.5% CO_2, 5% O_2/9% CO_2) over atmospheric air (21% O_2/0.05% CO_2) condition (~30%) (Fig. 1A). A similar phenomenon occurred with CFCs whose optimal recovery was obtained with 5% O_2/9% CO_2 gas mixture (62 ± 13% of CFCs relative to D-0 if preserved in 5% O_2/9% CO_2 vs. >25 ± 8% preserved in air and 25–40% in other hypoxic/hypercapnic gas mixtures tested). For this reason, we continued our study with 5% O_2/9% CO_2 only (Fig. 1A).

Viable cell number in hypoxia/hypercapnia remains stable from 5 to 10 days at 4°C, whereas it declines in the same time lapse in air (data not shown). Thus, hypoxia/hypercapnia seems to provide protective environment that reduces cellular degradation in hypothermia.

Full culture expansion potential of CD34$^+$ cells incubated in hypoxia/hypercapnia at 4°C and subsequently transferred to physiological temperature (37°C) cytokine-supplemented

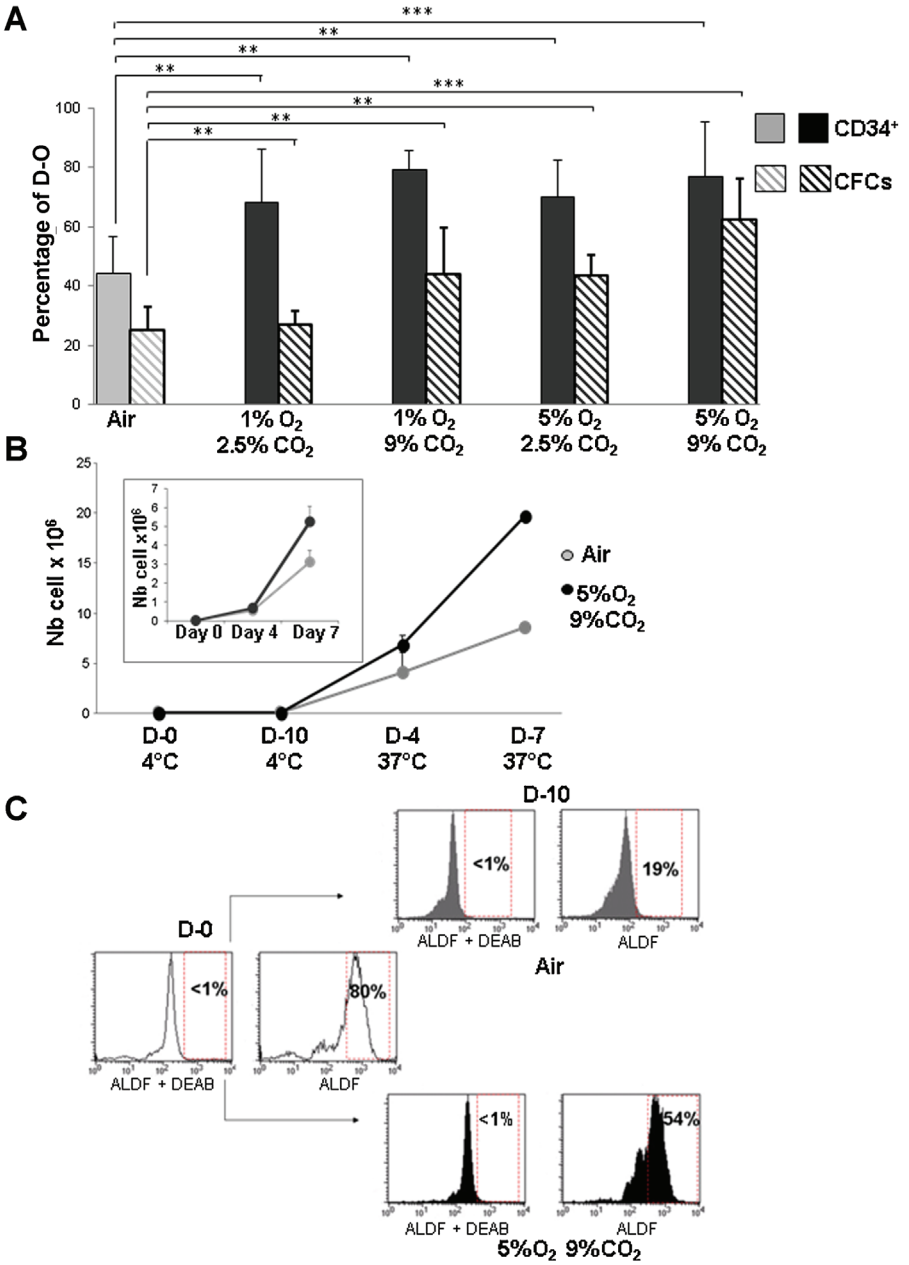


Fig. 1. Conservation of hematopoietic progenitors in hypothermia. A: Maintenance of $CD34^+$ cells and committed progenitors (CFCs) under different gas mixtures after 10 days incubation at 4°C. The bars represent average percentage of $CD34^+$ (filled bars) or CFCs (striped bars) preserved after 10 days incubation at 4°C relative to D-0. The data are presented as a mean ± SD of 19 independent experiments. Asterisks indicate a significant difference at $P < 0.01$ (**) and $P < 0.005$ (***), Wilcoxon test. B: Overall cell expansion of the remaining cells from 10^5 $CD34^+$ cells after 10 days incubation of cells in air (gray line) or in 5% O_2/9% CO_2 (black line). Preserved cells were cultivated for 10 days in the presence of growth factors (SCF, MGDF, G-SCF, 100 ng/ml) at 37°C. Inset: Expansion potential of 10^4 $CD34^+$ remaining after 10 days incubation at 4°C in air or in 5% O_2/9% CO_2 and cultivated at 37°C in same conditions as above. Results are presented as a mean of total cell number ± SD of four independent experiments. C: Analysis of ALDH activity. Histograms represent fluorescence intensity of $CD34^+$ D-0 cells (white) or preserved cells after 10 days (D-10) storage in air (gray) or in hypoxic/hypercapnic (5% O_2/9% CO_2) mixture (black), treated with substrate ALDF in the presence of the ALDH inhibitor DEAB or with ALDF alone. A representative of four independent experiments is shown.

culture turns out to be twofold greater than of the cells initially incubated in air (Fig. 1B). Also, relative cell expansion potential estimated per 10^4 $CD34^+$ cells harvested after cold storage (Fig. 1B inset) is approximately twofold higher if the cells were recovered from hypoxia/hypercapnia. These results suggest an improved functional maintenance of the primitive progenitors in this condition.

We also measured ALDH activity, a metabolic marker of primitive progenitors and stem cells (Hess et al., 2006). Comparing to D-0 $CD34^+$ cells (~80% expressing active ALDH), 10 days incubation in hypothermia remarkably reduced ALDH activity in air (~20%). In contrast, cells expressing active ALDH are better preserved in hypoxia/hypercapnia (~50%) (Figs. 1C and S1).

Hypoxia/hypercapnia preserves SRC activity of CD34$^+$ cells in hypothermia

When 10^3 D-0 CD34$^+$ cells or their D-10 equivalent (content of the same volume fraction as the one containing 10^3 CD34$^+$ cells at D-0; the number of CD34$^+$ cells injected per mouse at D-10 varied between 440 and 800, depending on experimental condition and individual experiment variation) are injected in NOD-scid IL2rγ^{null} (NSG) mice, human cells were detected in some, but not all mice 6 weeks later. Thus, this very low dose of cells allows a semi-quantitative comparison of the repopulating potential of the cells. Based on the proportion of NSG mice positive for human CD45 antigen in their bone marrow (BM) (SRC-$_{cd}$), it is evident that incubation in hypoxia/hypercapnia maintained full engraftment potential of D-0 cells, while in air only half of this potential was maintained (Fig. 2A).

Incubation in hypoxia/hypercapnia preserves not only the number of repopulating cells with short-term reconstitution activity (subpopulation SRC-$_{cd}$) (estimated on the basis of proportion of positive mice) but also their full proliferative

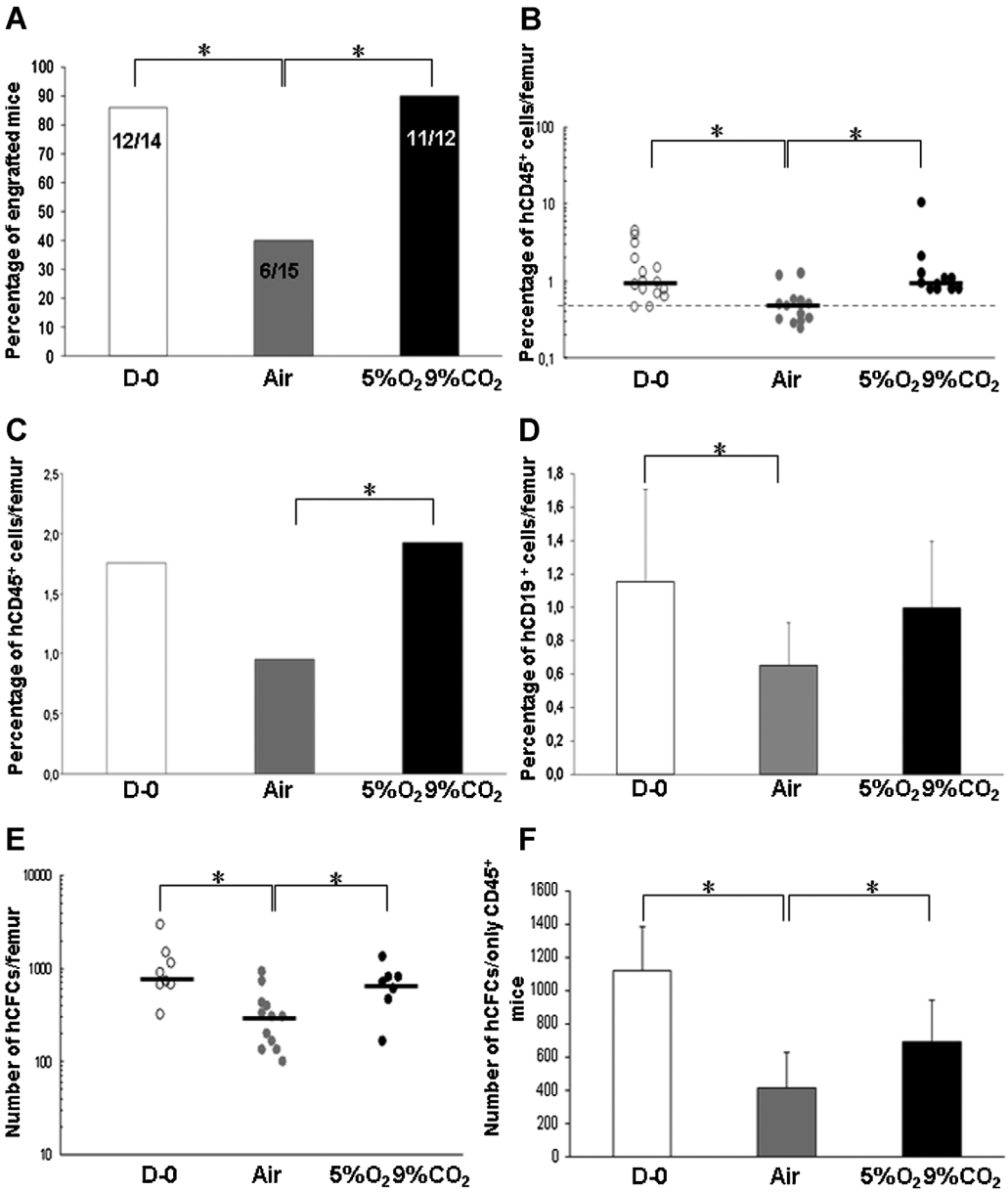


Fig. 2. SRC activity of the preserved CD34$^+$ in hypothermia. A: Frequency of the mice positive for human cord blood cell engraftment in their bone marrow (human CD45$^+$ chimerism >0.5%). The activity of SRC was detected on the basis of human CD45$^+$ chimerism in bone marrow of NSG mice 6 weeks postinjection of 10^3 steady-state CD34$^+$ cord blood cells (D-0) or their D-10 equivalent incubated in 5% O_2/9% CO_2 or in air. In all panels white bars or circles correspond to the effect observed after injection of D-0 or CD34$^+$ cell incubated 10 days 5% O_2/9% CO_2 (black bars or circles) or in air (gray bars or circles). B: Femoral bone marrow labeling with human CD45$^+$ antibody (each mouse represented by one circle). Median values are indicated by dashed lines. C: Average percentage of human CD45 chimerism in positive mice. D: Average number of human CD19 chimerism in CD45$^+$ mice. E: Human CFCs content per femur (each mouse represented by one circle). F: Average number of human CFCs in the CD45$^+$ mice. Cumulative results from four independent experiments are presented. Asterisks indicate a significant difference at $P < 0.05$ (*) estimated by χ^2 test (A) or Mann–Whitney test (B–D).

capacity according to the level of human CD45 chimerism (%) in the mouse BM which is the same as in mice injected with D-0 cells (Figs. 2B and S2). Again, a significant decrease in chimerism level was found in air condition (Figs. 2B and S2). This is even more obvious if the mean percentage of $CD45^+$ cells per femur of engrafted mice is presented (Fig. 2C); whereas prolonged hypothermia in air resulted in a decrease of lymphoid differentiation capacity (CD19) of stem cells (SRC), 5% O_2/9% CO_2 atmosphere maintained it well (Figs. 2D and S2).

If the analysis is based on the number of human CFCs in the murine BM generated during 6 weeks after the graft (Figs. 2E and F), it is evident that incubation in hypoxia/hypercapnia preserves a SRC sub-population ($SRC-_{CFC}$) activity similar to that of D-0 cells. On the other hand, in air condition, the capacity of $SRC-_{CFC}$ to generate CFCs in BM of transplanted mice is significantly lower with respect to D-0 cells and D-10 cells in hypoxia/hypercapnia.

Physiological status of preserved $CD34^+$ cells in hypothermia

In order to explain this beneficial effect of hypoxia/hypercapnia we analyzed physiological parameters critical for maintenance of cell homeostasis in prolonged hypothermia.

Analysis of the cellular energetic status of cells revealed that ATP amount/per viable cell (0.6 ± 0.2 pg/cell) after 10 days incubation at 4°C dropped severely comparing to D-0 levels (fivefold), as expected (Boutilier, 2001), irrespectively of the conservation condition (Fig. 3A).

Furthermore, as a functional indicator of the maintenance of plasma membrane, we estimated its potential (Mann and Cidlowski, 2001). FACS analysis revealed that D-0 $CD34^+$ cells had low DiBAC4(3) fluorescence (Fig. 3B), indicating their ability to exclude the dye, that is, to keep their membrane potential intact. After 10 days in hypothermia, we detected an increase of DiBAC4(3) fluorescence as a result of the plasma membrane depolarization. This was, however, significantly less pronounced in hypoxia/hypercapnia than in air (Fig. 3B).

Next, as an indicator of the integrity of mitochondrial membrane, we analyzed its potential (Kroemer et al., 2007). Prolonged hypothermia induces collapse in mitochondrial membrane potential when compared to D-0 level (Fig. 3C) (estimated by fluorescence decrease of mitochondrial potential-sensitive dye TMRM). Again, this was significantly less pronounced in hypoxia/hypercapnia than in air. Further, we determined formation of ROS, a key mediator of the cold induced injuries and apoptosis after re-warming (Rauen and de Groot, 2004). First, we analyzed the level of the cellular oxidative stress by commonly used probe H_2DCF-DA (Jang and Sharkis, 2007; Piccoli et al., 2007). The ROS content increased in hypothermia in comparison to D-0, to reach, after 10 days, the similar levels in two conservation conditions (Fig. 3D). However, significantly more necrotic and apoptotic cells (Table S1) were detected in air. Considering the limitations of H_2DCF-DA assay (Karlsson et al., 2010; Kalyanaraman et al., 2012) we further determined critical ROS implicated in the hypothermic-induced cellular injuries, H_2O_2 and its precursor, $O^{\cdot-}_2$ with more specific techniques. Using Amplex red as an efficient probe for the detection of the extracellular H_2O_2 in the presence of the horseradish peroxidase, we estimated H_2O_2 released by the preserved $CD34^+$ cells. Our results showed that H_2O_2 production increased during the cold storage, in the same manner under both conservation conditions with respect to D-0 $CD34^+$ cells (Fig. 3E). In contrast, detection of $O^{\cdot-}_2$ level by ERP after 10 days incubation in hypothermia showed greater value in the hypoxia/hypercapnia than in air (Fig. 3F). Thus, hypoxia/hypercapnia improves maintenance of viable cells and induces less apoptosis despite the production of deleterious ROS.

Bioenergetic analysis

To evaluate the effect of hypoxia/hypercapnia on the energetic metabolism, we monitored the OCR and ECAR in D-0 and D-10 $CD34^+$ cells. Analyzing mitochondrial functions by the mitochondrial stress test, conservation in hypothermia did not change basal OCR of $CD34^+$ cells regardless of the condition (Figs. 4A,B and S3A). In contrast, basal OCR/ECAR ratios in $CD34^+$ cells preserved in hypoxia/hypercapnia was significantly decreased comparing to that observed in air and diminished was similar to the values of D-0 cells (Figs. 4C and S3A–C). Addition of oligomycin, a F0/F1 ATPase inhibitor, induced a drop in OCR (measurements 3 and 4). The mean basal levels of OCR minus the mean of the two values following oligomycin treatment represent the amount of oxygen consumption that is linked to the ATP synthesis in the mitochondria. These results suggested that energy supply in hypoxia/hypercapnia conserved cells rely less on the mitochondrial respiration (Figs. 4A and B). Under this condition we detected reduced maximal mitochondrial respiration after DNP addition (uncoupling agent, measurements 5 and 6) in comparison to air (Fig. 4A). Observed responses in hypoxia/hypercapnia preserved cells were similar to those obtained in D-0 $CD34^+$ cells (Figs. 4B and S3A).

Performing the glycolysis stress test, we observed that average ECAR values detected in glucose-free medium and after sequential injection of glucose and oligomycin, respectively (Figs. 4D and S3D), revealed that, in general, hypothermia reduced the glycolytic functions in comparison to D-0 $CD34^+$ cells (Fig. 4E). 2-Deoxy-glucose sensitive ECAR decline indicates that observed ECAR changes are due mainly to glycolysis. (Figs. 4D,E and S3D). Also, we compared different conservation conditions and determined that cells preserved in hypoxia/hypercapnia had small but significantly greater glycolytic activity than in air (Figs. 4D and E).

Altogether, these results indicate that while conservation in air promotes oxygen consumption in $CD34^+$ cells, hypoxia/hypercapnia tends to preserve energetic profile of D-0 $CD34^+$ cells.

Hypercapnia has a critical role in the cell protection against cold stress

Our results showed that hypercapnia alone enhanced maintenance of $CD34^+$ cells and CFCs in hypothermia as hypoxic/hypercapnic atmosphere did. In contrast, hypoxia alone had no effect on the maintenance of CFCs and slightly enhanced $CD34^+$ cell maintenance (Fig. 5A), suggesting the primary role of hypercapnia. This effect is associated with CO_2-induced decrease of pH in the conservation medium and intracellular environment (Fig. 5B). Next, we tested if the observed beneficial effects of hypercapnia could be mimicked by the acid loading. An acidic conservation medium (pH 6 ± 0.3) improved significantly the maintenance of $CD34^+$ cells in air, but had no effect on the cell survival in hypoxia/hypercapnia (Fig. 5C). Nevertheless, an apoptosis assay revealed that a slightly acidic conservation medium ($6.3 < pH < 6.7$) in air still induced more early apoptotic and late apoptotic/necrotic cells than in hypoxia/hypercapnia (acidified or not) (Fig. 5D). Altogether, these results suggest that a hypercapnia-induced decrease of the pH of the medium is an important mechanism, but not the only one explaining cellular protection in hypothermia in hypoxic/hypercapnic environment.

Proteomic analysis

Proteomic analysis identified 147 proteins from D-0 and D-10 $CD34^+$ cells preserved in various conditions air, hypoxia/

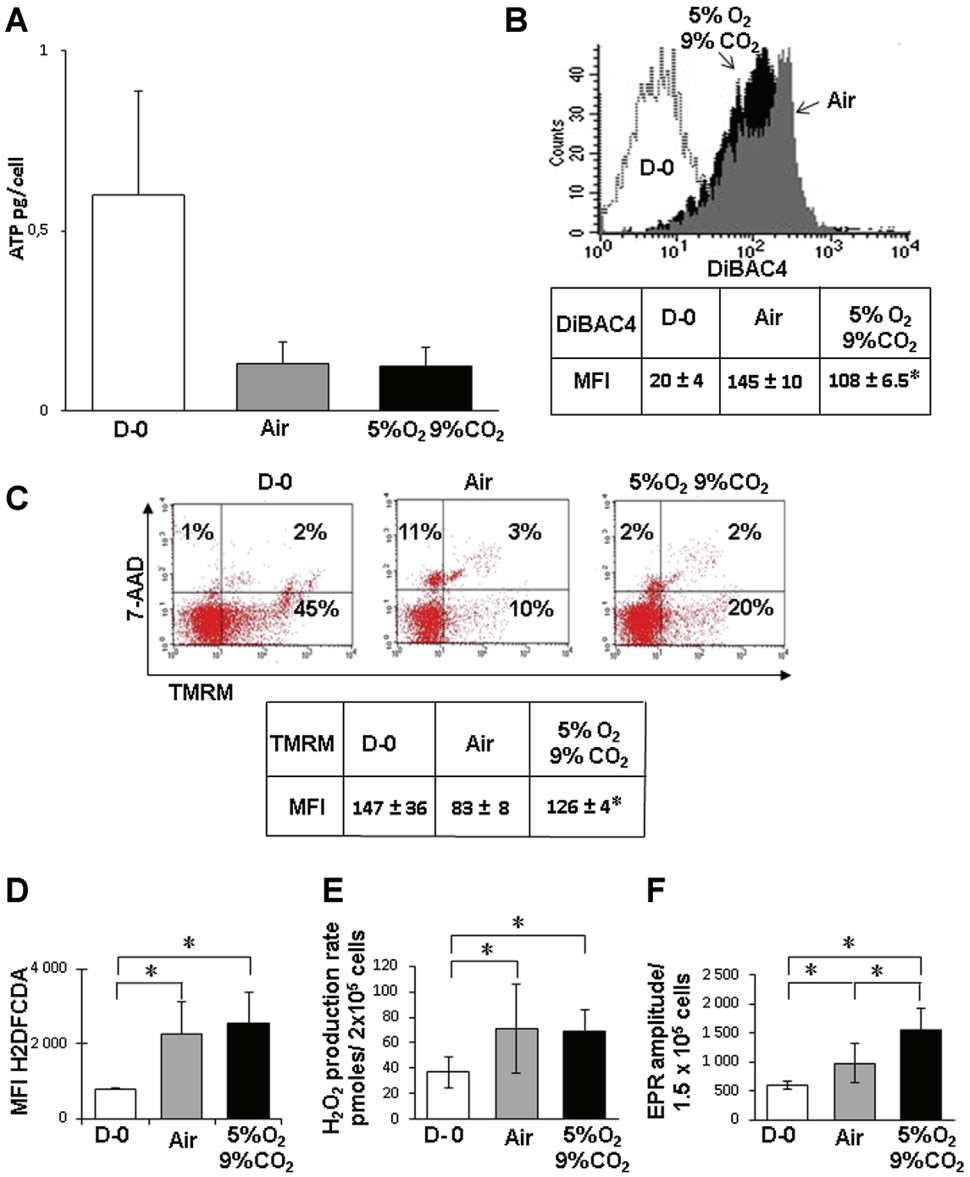


Fig. 3. Physiological characteristics of preserved CD34$^+$ cells at 4°C in various gas mixtures. The measurements of all examined physiological parameters were performed on D-0 (white symbols) or CD34$^+$ cells after 10 days incubation in air (gray symbols) or in 5% O_2/9% CO_2 (black symbols). A: ATP content is presented as the mean ± SD of four independent experiments. B: Plasma membrane potentials were estimated by fluorescence of DiBAC4(3) presented in the histogram. One representative of four independent experiments is shown. DiBAC4(3) mean fluorescence intensity (MFI) is presented as the mean ± SD of four independent experiments. In parts B and C asterisks indicate a significant difference at $P < 0.05$ (*), between conditions hypoxia/hypercapnia versus air estimated by Wilcoxon test. C: Dot plots represent TMRM fluorescence as the measure of mitochondrial membrane potential. 7-AAD viability marker was added to exclude the dead cells. One representative of four independent experiments is shown. TMRM MFI is presented as the mean ± SD of six independent experiments. D: The bars represent the level of ROS estimated by the MFI of H_2DCF-DA, as mean ± SD of four independent experiments. E: H_2O_2 measurements using Amplex red. Values are presented as the mean ± SD of the four independent experiments. F: $O^{\cdot -}_2$ level measured by EPR spectrometry. Data are expressed as amplitude of the pics out of 1.5×10^5 CD34$^+$ cells. Values are shown as the means ± SD of four independent experiments. Asterisks indicate a significant difference at $P < 0.05$ (*), Wilcoxon test.

hypercapnia, hypercapnia, or hypoxia only (Table S2). The best overall protein conservation compared to D-0 CD34$^+$ cells was observed in hypercapnia only and hypoxia/hypercapnia storage conditions. After incubation in these conditions, in comparison to air and hypoxia only, a higher number of proteins with unchanged expression and a lower number of proteins with decreased expression was detected with respect to the identified part of D-0 CD34$^+$ cell proteome (Fig. 6A). Furthermore, proteomic analysis highlighted: (i) proteins whose expression is not dependant on the gas phase condition, and (ii) proteins whose expression was specifically modulated depending on the condition (Fig. 6B).

The group of proteins insensitive to hypothermia (35 proteins, 24% of the total number detected) contained glycolytic enzymes (triosephosphate isomerase, fructose-bisphosphatase, aldolase, glyceraldehyde-3-phosphate dehydrogenase), proteins involved in the regulation of cell structure and motility (actin, myosin-9, filamin-a, profilin-1,

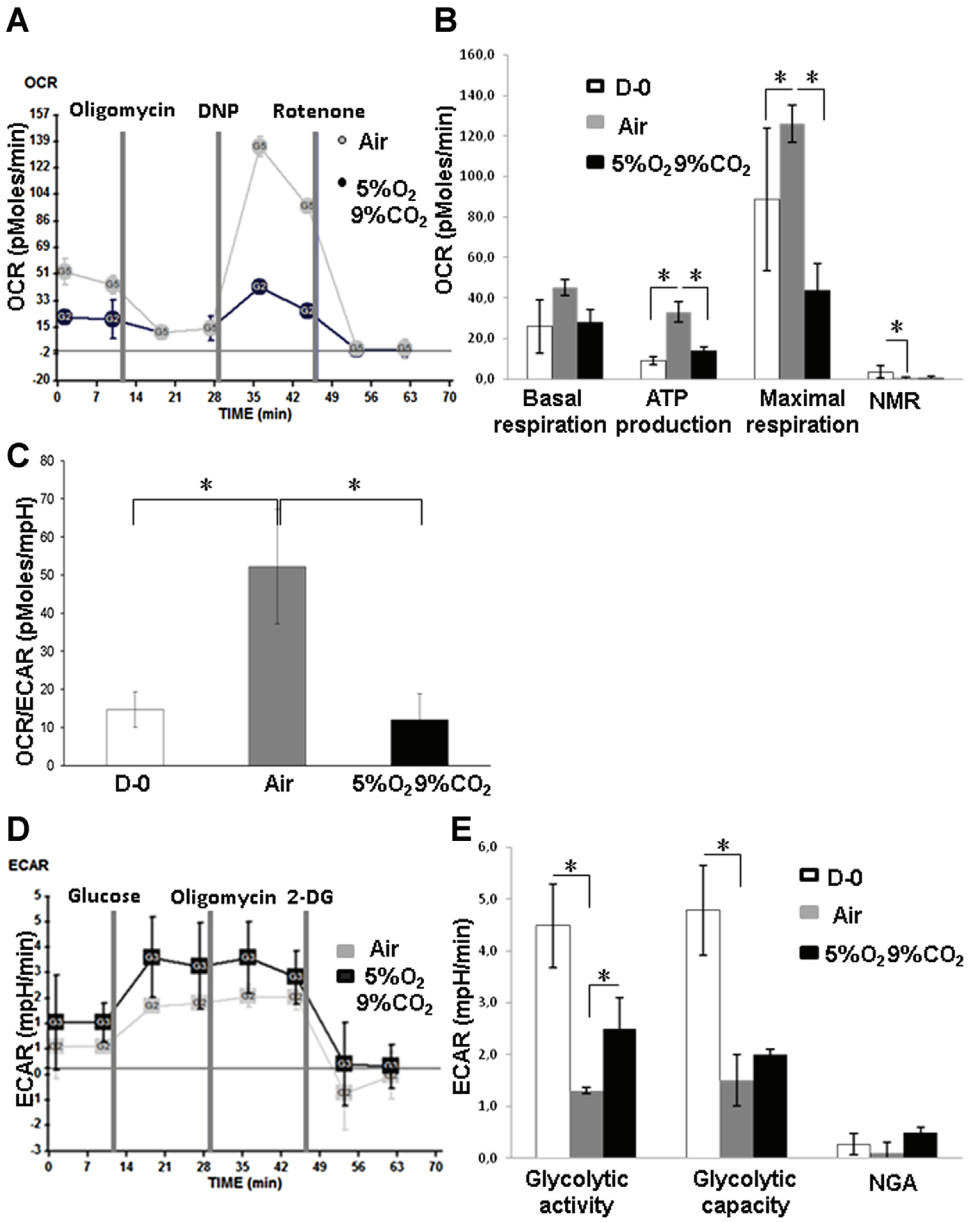


Fig. 4. Bioenergetic profile of preserved cells. Results obtained by mitochondrial stress test are illustrated in parts A–C, and by glycolysis stress test in parts D,E (see the Materials and Methods Section). A: OCR was determined by XF analyzer without (measurements 1 and 2) or after sequential injection of three mitochondrial inhibitors: oligomycin (1 μM) (measurement 3 and 4); 2,4 DNP (100 μM) (measurements 5 and 6); rotenone (1 μM) (measurements 7 and 8). The spots represent individual XF analyzer measurements. Modulators were injected after measurements 2,4,6, respectively in all experiments. B: The bars represent average basal respiration (mean of OCR values measurements 1 and 2), ATP production (mean basal OCR minus the mean of the two values following oligomycin treatment), maximal respiration (mean of OCR values, measurements 5 and 6) and non-mitochondrial respiration (NMR) (mean of OCR values, measurements 7 and 8). C: Average OCR/ECAR ratio for D-0 (white bar) and CD34$^+$ cells preserved in hypoxia/hypercapnia (black bar) or in air (gray bar). D: ECAR was determined in a glucose-free medium (measurements 1 and 2) or after sequential injection of: glucose (10 mM) (measurements 3 and 4); oligomycin (1 μM) (measurements 5 and 6); 2-deoxy-glucose (2DG) (100 mM) (measurements 7 and 8). The squares represent individual XF analyzer measurements. E: The bars represent average initial ECAR (mean of ECAR values measurements 1 and 2), glycolysis and glycolytic capacity (mean of ECAR values following injection of glucose (measurements 3 and 4) or oligomycin treatment (measurements 5 and 6) minus initial ECAR value, respectively) and non-glycolytic acidification (NGA) (mean of ECAR values, measurements 7 and 8). Representation as in C. Parts A and D represent one of four independent experiments. Data at parts B,C,E are shown as the mean ± SD of four independent experiments. Asterisks indicate a significant difference at $P < 0.05$ (*), Wilcoxon test.

thymosin), in redox regulation (peroxiredoxin-1, glutathione S-transferase), and chaperones (HSP 70, mitochondrial protein 1A/1B 10 kDa) (Table S2). In contrast, another group of proteins sensitive to hypothermia included 48 proteins (33% of the total number detected): chaperones (HSP 90-beta, HSP 71, HSP 60, nucleophosmin), ribonucleoproteins, enzymes involved in ATP synthesis and metabolism (ADP/ATP translocase 2, ATP synthase subunits), redox regulation (thioredoxin, catalase, superoxide dismutase [Cu-Zn], peroxiredoxin-2, thioredoxin-dependent peroxide reductase),

A

% D-0	CD34+	CFC
Air	38 ± 3.2	26 ± 10
HH	79 ± 2.8***	54 ± 15**
Hyper	87 ± 3.5***	70 ± 18***
Hypo	52 ± 3.2**	31 ± 10

B

	CD34+ D-0	Air D-10	5%O_2 9%CO_2 D-10
pHe		7,5 ± 0,1	6,4 ± 0,1*
pHi	7,4 ± 0,1	7,3 ± 0,1	6,6 ± 0,4*

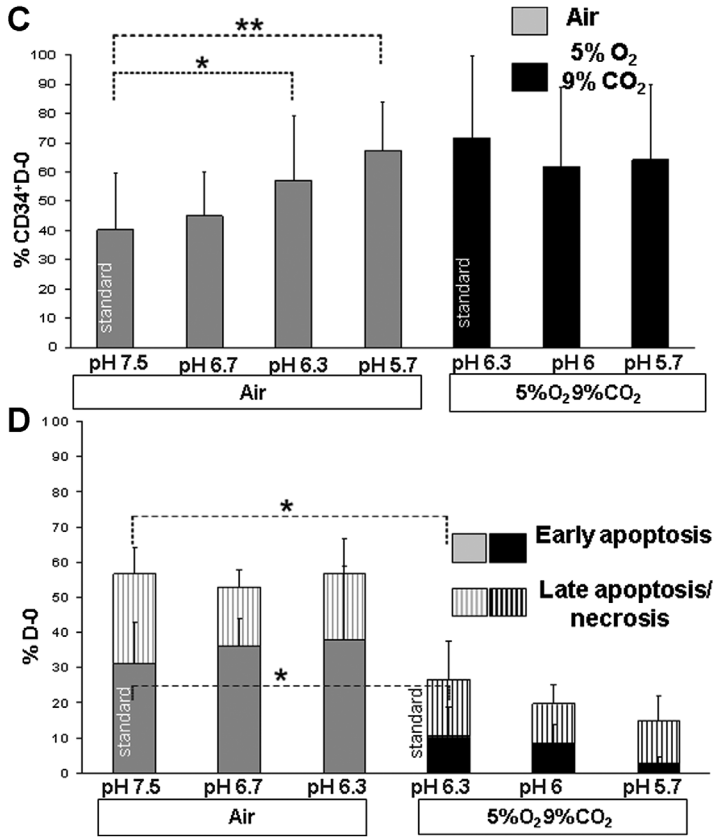


Fig. 5. Influence of pH on the cell maintenance in hypothermia. A: Maintenance of $CD34^+$ cells or committed progenitors (CFCs) after 10 days incubation in air, or hypoxia (5% O_2)/hypercapnia (9% CO_2) or hypercapnia (20% O_2/9% CO_2) or hypoxia (5% O_2/0% CO_2). Data are presented as the mean percentage of preserved cells relative to D-0 ± SD of five independent experiments. Asterisks indicate a significant difference at $P < 0.01$ (**) and $P < 0.005$ (***) versus D-0 $CD34^+$ cells and CFCs estimated by Wilcoxon test. HH, hypoxia/hypercapnia; hyper, hypercapnia; hypo, hypoxia. B: pH measurement. Intracellular (pH_i) was evaluated in D-0 $CD34^+$ cells and in cells incubated 10 days in air or hypoxia/hypercapnia. Extracellular pH (pH_e) of the conservation medium was determined after 10 days incubation in the corresponding condition. Values are presented as a mean ± SD of 12 or 4 independent experiments for the measurement of pHe or pHi, respectively. Asterisks indicate a significant difference at $P < 0.05$ (*), between conditions hypoxia/hypercapnia versus air estimated by Wilcoxon test. C: pH dependence of the $CD34^+$ cells preservation. Bars represent $CD34^+$ cells preserved in the medium with standard or lower pH values when incubated in air (gray bars) or in hypoxic/hypercapnic gas mixture (black bars) in hypothermia for 10 days. Data are presented as the mean percentage of preserved cells relative to D-0 ± SD of 12 independent experiments. D: Apoptosis pH dependence. Bars represent the occurrence of early ($AnnV^+/PI^-$, filled bars) and late apoptotic/necrotic cells ($AnnV^+/PI^+$, striped bars) in the population of $CD34^+$ cells treated as B). Data are presented as the mean percentage of apoptotic cells relative to D-0 ± SD of five independent experiments. Asterisks indicate a significant difference at $P < 0.05$ (*), $P < 0.01$ (**), and $P < 0.005$ (***), Wilcoxon test.

cell structure and motility (myosin light polypeptide 6, myosin regulatory light chain 12a, annexin A5, keratin, type II cytoskeletal 6b, talin-1, tubulin) (Table S2).

The third group consisted of proteins whose expression was augmented non-specifically with respect to D-0 levels. Among these we identified histones, apoliprotein and polyubiquitin-C (Table S2).

Finally, some proteins appeared insensitive to hypothermia and were specific to hypoxia/hypercapnia and hypercapnia only. We believe that these proteins particularly contribute to the observed protective effects. In this category we identified some enzymes involved in glycolysis and TCA cycle, DNA repair, cell signalling (calmodulin), cytoskeleton regulation (stathmin, Rho GDP-dissociation inhibitor 2, Ras GTPase-activating-like protein), cell motility structure (moesin, Ras-related protein Rap-1A, plastin) chaperones (HSP 90-alpha). In addition, we detected proteins whose expression is specifically increased in hypoxia/hypercapnia and hypercapnia with respect to D-0 (e.g., a serpin family member, leukocyte elastase inhibitor) (Fig. 7). Interestingly, serpin protein family is implicated in the long-term survival of hibernating animals in hypothermia (Boutilier, 2001).

Discussion

Our study indicates that hypoxia/hypercapnia in hypothermia preserves all cell compartments in the $CD34^+$ cell population necessary for successful engraftment. Furthermore, in hypoxia/hypercapnia condition we obtained a fivefold increase of the period for the liquid storage of CB hematopoietic cells over the currently accepted limit (48 h), which maintains complete cell functionality and viability with the respect to D-0.

In order to estimate functional maintenance of primitive hematopoietic progenitors within $CD34^+$ cell population we evaluated their hallmark characteristic, the expansion capacity in culture at physiological temperature (37°C) (Duchez et al., 2012). This feature is known to be abrogated upon cold storage, which leads to apoptosis upon re-warming. The increased expansion capacity of $CD34^+$ cells stored in hypoxia/hypercapnia (Fig. 1B) indicates that our method circumvents

A

	AIR	HH	Hyper	Hypo
down	79	68	72	81
up	8	7	9	9
nc	60	72	66	57

B

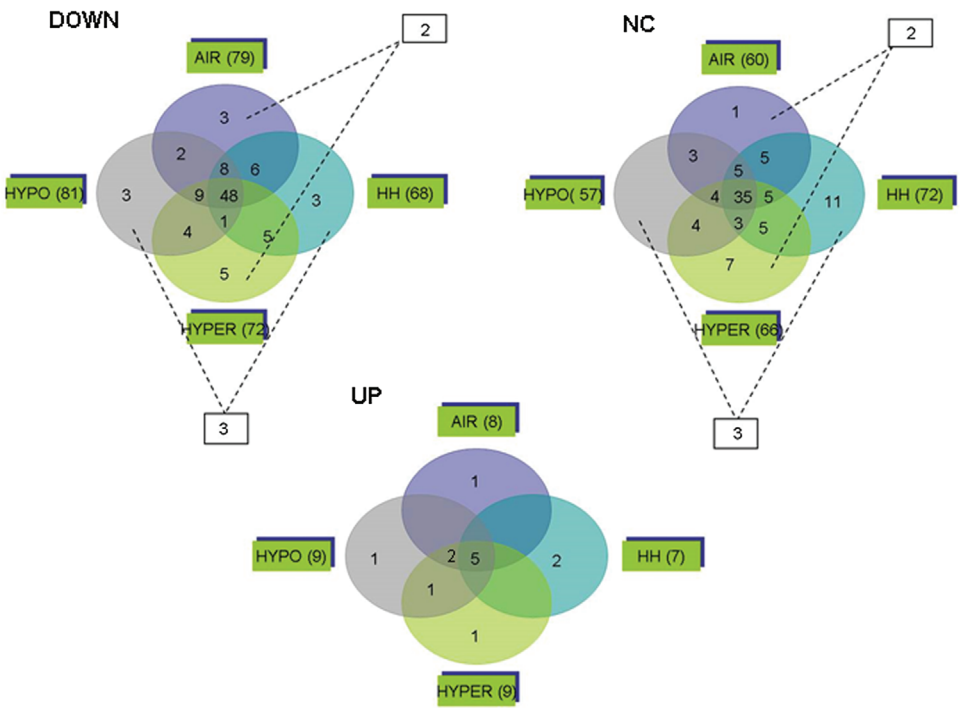


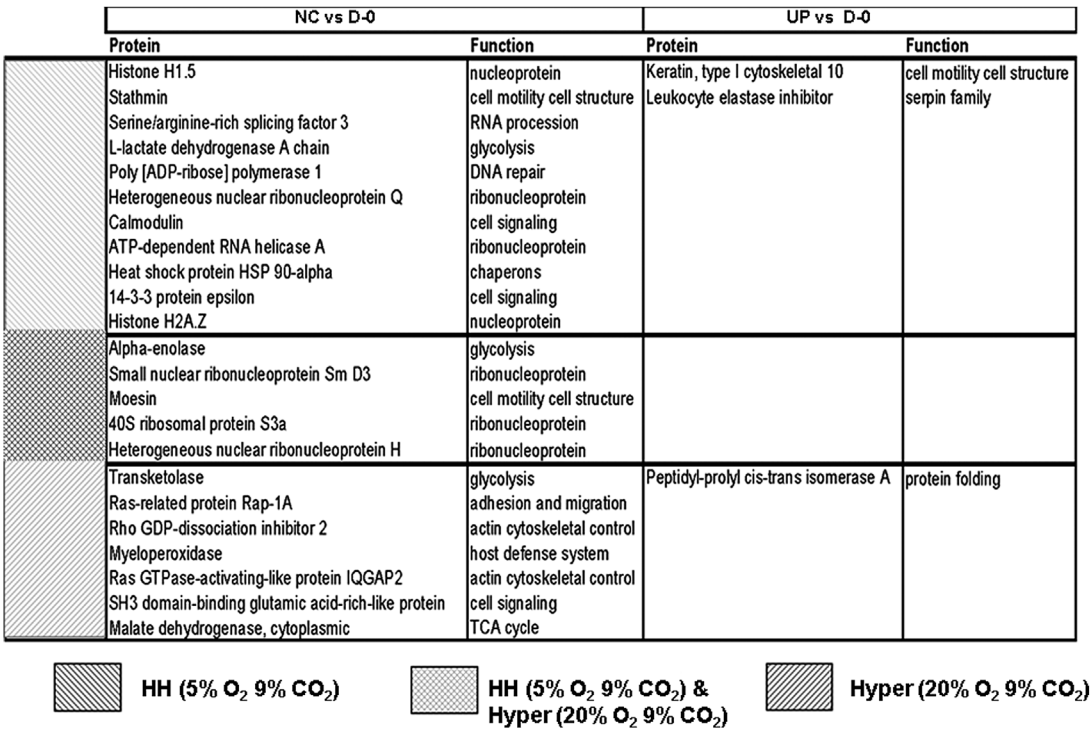
Fig. 6. Proteomic analysis of CD34$^+$ cells preserved in hypothermia. A: Number of proteins identified in CD34$^+$ cells incubated 10 days at 4°C in air, hypoxia/hypercapnia, hypercapnia, or hypoxia relative to D-0 34$^+$ (NC—not changed). B: Venn diagram of identified proteins.

hypothermic damages and thus enables maintenance of primitive progenitors completely capable of ex vivo expansion.

Finally, the maintenance of very rare CD34$^+$ primitive stem cells with capacity for in vivo short-term reconstitution activity was evidenced by an SRC assay (Ivanovic et al., 2011; Hammoud et al., 2012). These findings indicated that maintenance of the proliferative and lymphoid differentiation capacity of SRC in hypothermia was completely accomplished by hypoxia/hypercapnia (Fig. 2). Consistently, hypoxia/hypercapnia induced preservation of the cells expressing ALDH thus showing maintenance of the functional primitive hematopoietic cells (Fig. 1C) (Kastan et al., 1990; Storms et al., 2005; Hess et al., 2006). It remains to be confirmed if hypoxia/hypercapnia condition exhibits the same beneficial effect on long-term repopulating stem cells preservation in hypothermia.

Analyzing the physiological aspects of this phenomenon, we showed that these beneficial effects of hypoxia/hypercapnia are based on a better maintenance of the properties determining sensitivity to hypothermia, integrity of the plasma membrane and functionality of mitochondria (Fig. 3).

With regards to the mitochondrial function, it is known that ROS produced during the hypothermic storage are the principal cause of mitochondrial permeability transition connected to the loss of mitochondrial membrane potential and finally their function (Kerkweg et al., 2003). But, in our model, we showed that hypoxia/hypercapnia-mediated protection of the mitochondrial function (Fig. 3C) is not the consequence of a lower ROS production (Figs. 3D–F). These findings, consistent with those showing the maintenance of functional stem cell capacity during progressive in situ development of hypercapnia, hypoxia, and hyperthermia postmortem (Latil et al., 2012), suggested the existence of other mechanisms. Also, neither the potential pro-ROS effects of the pair CO_2/bicarbonate (Queliconi et al., 2013), nor iron-induced ROS formation in hypoxia/hypercapnia condition seem to impair the functional maintenance of stem and progenitors cells. In hypothermia, membrane ionic carriers are inactive and only non-charged forms of molecules are able to passively cross the membrane (Durand et al., 1998). This means that in the hypercapnic condition CO_2 diffuses across the membrane, enabling permanent load of carbonic acid and protons in the cytosol (Fig. 5B). This acidic environment inhibits loss of mitochondrial function as already shown (Kim et al., 2006). In addition, the hypoxic action improves cell conservation in hypothermia, resulting in less apoptosis upon transfer to 37°C (Rauen et al., 1999). Accordingly, the



NC vs D-0		UP vs D-0	
Protein	Function	Protein	Function
Histone H1.5	nucleoprotein	Keratin, type I cytoskeletal 10	cell motility cell structure
Stathmin	cell motility cell structure	Leukocyte elastase inhibitor	serpin family
Serine/arginine-rich splicing factor 3	RNA procession		
L-lactate dehydrogenase A chain	glycolysis		
Poly [ADP-ribose] polymerase 1	DNA repair		
Heterogeneous nuclear ribonucleoprotein Q	ribonucleoprotein		
Calmodulin	cell signaling		
ATP-dependent RNA helicase A	ribonucleoprotein		
Heat shock protein HSP 90-alpha	chaperons		
14-3-3 protein epsilon	cell signaling		
Histone H2A.Z	nucleoprotein		
Alpha-enolase	glycolysis		
Small nuclear ribonucleoprotein Sm D3	ribonucleoprotein		
Moesin	cell motility cell structure		
40S ribosomal protein S3a	ribonucleoprotein		
Heterogeneous nuclear ribonucleoprotein H	ribonucleoprotein		
Transketolase	glycolysis	Peptidyl-prolyl cis-trans isomerase A	protein folding
Ras-related protein Rap-1A	adhesion and migration		
Rho GDP-dissociation inhibitor 2	actin cytoskeletal control		
Myeloperoxidase	host defense system		
Ras GTPase-activating-like protein IQGAP2	actin cytoskeletal control		
SH3 domain-binding glutamic acid-rich-like protein	cell signaling		
Malate dehydrogenase, cytoplasmic	TCA cycle		

Fig. 7. Unchanged or upregulated proteins under hypoxia/hypercapnia and hypercapnia only versus D-0 CD34$^+$ cells. HH, hypoxia/hypercapnia; hyper, hypercapnia.

beneficial effect of hypoxia in our study enables better CD34$^+$ survival (Fig. 5A) as well as SRC–$_{CFC}$ preservation (data not shown).

Better survival of cells preserved in hypoxia/hypercapnia during cell recovery from the hypothermic stress is also facilitated by the fact that their energetic balance is less dependent on the oxidative phosphorylation (Fig. 4) (Murphy, 2009). However, actions of hypoxia/hypercapnia did not ensure the means for stable ATP turnover in hypothermia (Fig. 3A). This is in agreement with other data showing that energetic deficit in hypothermia (Boutilier, 2001; Brinkkoetter et al., 2008) is developed as the consequence of the exhaustion of the intracellular ATP by mismatch between ATP demands and decreased ATP synthesis.

We reported here that the proteins generally degraded in hypothermia are implicated in: (1) cell motility and maintenance of the cell structure; (2) protein biosynthesis, proteins processing, folding, ubiquitination; (3) cell signalling; (4) stress response and oxidative defense; (5) energetic metabolism; (6) transcription regulation; and (7) DNA repair.

Analysis of the protein group non-specifically tolerant to severe hypothermia indicated several important features. First, we identified the proteins implicated in the stability persistence of the cytoskeleton in hypothermia (actin, myosin-9, filamin-A, profilin-1, thymosin beta-4). Second, we reported that in contrast to mitochondrial ATP machinery production, glycolytic enzymes are more stable in hypothermia. Taken together these findings suggest that: (1) better expansion of cells after rewarming can be accomplished in medium favouring glycolytic energetic pathway, and (2) primitive hematopoietic progenitors achieve better hypothermic tolerance as they predominantly rely more on glycolytic machinery to satisfy their energetic demands (Simsek et al., 2010). In addition, in this group we detected some anti-oxidative defense enzymes and chaperones indicating that preserved cells maintain to some extent the properties to exhibit a protective physiological response during the stress response that ensues after rewarming.

Proteins whose expression was upregulated in all conditions (Table S2) indicate that their expression could be stimulated as part of protective stress response against the cold shock (e.g., ubiquitin and apoliprotein A) as it was showed previously (Epperson and Martin, 2002; Sonna et al., 2002; Van Breukelen and Martin, 2002; Martin et al., 2008).

In addition, we demonstrated that specific cold-protective action of hypoxic/hypercapnic and hypercapnia only conditions account for overall better maintenance of the part of proteome identified in this study (Fig. 6A). Importantly, we identified proteins implicated in DNA repair, cold-shock response, and preservation/repair of protein folding, control of microtubular stability, that potentially contribute to hypoxia/hypercapnia induced cold tolerance (Fig. 7) or to general maintenance of cell homeostasis (Slaughter and Black, 2003; Belletti and Baldassarre, 2011).

This study highlights the importance of the mitochondrial and plasma membrane integrity during hypothermic tolerance in the primitive hematopoietic cells as well as to several proteins potentially involved in the beneficial hypoxia/hypercapnia effect. The better maintenance of progenitor and stem cells in hypothermia results from several different mechanisms of hypoxia/hypercapnia operating via stabilization of specific proteins, acidification and maintenance of mitochondria integrity. These effects are not related to commonly measured ROS decrease since their sustained production was evident, but do interfere with decrease of intracellular labile iron that seems to be the most interesting mechanism related to HH action (work in progress).

These results inspired new design for a procedure aimed to improve functional preservation of hematopoietic stem and progenitor cells in CB stored at 4°C before cryopreservation. In fact, by collection of CB into gas non-permeable bags containing a nutritive medium supplied by antioxidants, the prevention of CO_2 leak and exposure to atmospheric O_2 concentration (i.e., the hyper-oxygenation since atmosphere O_2 concentration is 21%) were achieved. So, by exploiting the principle of "hypoxia/hypercapnia" (with respect to air storage condition in hypothermia) we realized the maintenance of functional committed progenitors and SRC D-0 values up to 72 h. Furthermore, these cell populations were better maintained after 72 h with our procedure than after 24 h applying the actual routine collection in gas-permeable bags without medium (Chevaleyre et al., 2014). We are currently working on translation of these principles into protocols for conservation of ex vivo expanded CB cells. This clinical scale procedure could have a big impact on CB collection/transport/storage practice and could improve functional potency of stored CB units, specifically in the countries with the mandatory periods between collection and freezing (up to 3 days).

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