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Vincent Praloran, Amélie Guitart, Christelle Debeissat, Francis Hermitte, Arnaud Villacreces, et al.. Very low oxygen concentration (0.1%) reveals two FDCP-Mix cells subpopulations that differ by their cell cycling, differentiation and p27KIP1 expression. Cell Death and Differentiation, 2010, 10.1038/cdd.2010.85 . hal-00560771

HAL Id: hal-00560771

https://hal.science/hal-00560771v1

Submitted on 30 Jan 2011

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Very low oxygen concentration (0.1%) reveals two FDCP-Mix cells subpopulations that differ by their cell cycling, differentiation and p27^{KIP1} expression

RUNNING TITLE: **Hypoxia reveals two FDCP-mix cell subsets**

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Abstract

Oxygen concentrations in bone marrow vary from 4% in capillaries to less than 0.1% in subendosteum where hematopoietic stem cells reside in specific niches. Culture at low O_2 concentrations (3, 1 and 0.1%) influences hematopoietic stem and progenitor cells survival, proliferation and differentiation, depending on their level of differentiation. Culture of human $CD34^+$ cells at low O_2 concentrations ($O_2 \leq 3\%$) maintains stem cell engraftment potential better than at 20% O_2 (NOD/Scid xenograft model). In contrast, progenitors disappear from cultures at/or below 1% O_2 concentrations. A very low O2 concentration (0.1%) induces CD34+ quiescence in G_0. The exploration of molecules and mechanisms involved in hematopoietic stem and progenitor cells' quiescence and differentiation related to low O2 concentrations is unfeasible with primary $CD34^+$ cells. Therefore, we performed it using murine hematopoietic non leukemic FDCP-Mix progenitor cell line. The culture of the FDCP-Mix line at 0.1% O_2 induced in parallel G_0 quiescence and granulo-monocytic differentiation of most cells, while a minority of undifferentiated self-renewing cells remains in active cell cycle. Hypoxia also induced hypophosphorylation of pRb and increased the expression of $p27^{KIP1}$, two proteins that play a major role in the control of G_0 and G_1 to S phase transition.

Keywords: hematopoiesis, hypoxia, cell cycle, quiescence, differentiation, progenitors, p27, FDCP-Mix

Abbreviations: O_2, Oxygen; HIF-1, hypoxia-inducible factor-1; Epo, Erythropoietin; VEGF, Vascular Endothelial Growth Factor; HSC, Hematopoietic stem cell; PI, Propidium Iodide; CFSE, carboxyfluorescein diacetate succinimidyl diester; MFI, Mean Fluorescence Intensity; FDCP-Mix, Factor Dependent Cell Paterson; FCM, flow cytometry; CRA, Culture-Repopulating Ability; CFC, Colony Forming Cell; LC, Liquid Culture; KIP, Kinase Inhibitor Protein; CDK, Cyclin Dependant Kinase.

Introduction

Physiological oxygen (O_2) concentrations in tissues are much lower than in atmospheric air (20%), ranging from 14% in pulmonary alveoli to less than 1% in several organs (1). Numerous publications demonstrate the major role of variations of O_2 concentration in the responses of normal cells, transformed cells and leukemic cells from primoculture or from continuous cell lines (2-8). Low O_2 concentration induces apoptosis or cell survival, proliferation or quiescence, self renewal or differentiation depending on the degree of hypoxia, the cell type and the level of differentiation.

Response to hypoxia is largely dependent of hypoxia-inducible factor 1 (HIF-1), a heterodimeric transcription factor. At O_2 concentrations lower than 5%, the HIF-1α subunit is no longer degraded by the proteasome (9, 10) and dimerizes with HIF-1β. The active complex then induces the expression of numerous genes including those of growth factors (Erythropoietin (Epo) and Vascular Endothelial Growth Factor (VEGF)), transcription factors and cell cycle proteins (11-14).

In bone marrow, hematopoietic cells are permanently exposed to O_2 concentrations ranging from 4% in capillaries to less than 0.1% in endosteal areas (15-17) where the most primitive stem cells reside (18, 19). Hematopoietic stem cells (HSC) are better preserved in cultures at low O_2 (1 to 3 %) than at atmospheric O2 concentration (20%) (5, 20, 21). We previously showed that culture at 0.1% O_2 prevents cord blood G_0 $CD34^+$ cells entry in active cell cycle and induces those in active proliferation to re-enter in G_0 (22). The effects of low O_2 concentration on the survival, proliferation and differentiation of normal and leukemic hematopoietic progenitors are more controversial. Indeed, in some articles a pro-differentiative effect (6, 23-25) was pointed out while in others it was suggested that oxygen levels below 1% block cell proliferation and induce their rapid disappearance from cultures, probably by inducing apoptosis (4, 21, 26, 27).

Culture at low O_2 concentration (0.5%) causes cell cycle arrest in G_1 of mouse embryonic fibroblasts and splenic B lymphocytes by inducing Rb hypophosphorylation and $p27^{KIP1}$ upregulation (28, 29). All these results led us to explore the effects of low O_2 concentration on the proliferation and differentiation of hematopoietic progenitors. Extensive protein studies using normal human or murine primoculture progenitor cells were quite unfeasible for two reasons: heterogeneity of the $CD34^+$ cell population and difficulty of obtaining them in large numbers. Since the murine cell line FDCP-Mix, which is less heterogeneous, has major features of normal hematopoietic progenitors, we tested its response to hypoxia. Indeed, FDCP-mix cells have a normal karyotype, are non-leukemic, strictly depend on IL-3 for their survival and proliferation, and differentiate towards different myeloid lineages when cultured with other cytokines (30). Varying the concentrations of O_2 from 20 to 0.1% in cultures of FDCP-mix cells had a time -dependent effect on their proliferation and cell cycle, with cells reaching quiescence (G_0) after 72h of culture at 0.1% O_2. Then we used these cells to analyze the self renewal/proliferation/differentiation balance and the variations of $p27^{KIP1}$ levels and pRb phosphorylation, two proteins potentially involved in G_0 cell cycle arrest.

Results

Low O_2 concentration inhibits FDCP-Mix cell growth

Low O_2 concentration induced a time-dependent FDCP-Mix growth inhibition (fig 1a). The number of living cells at 24h was not significantly different in 20% O_2 vs. 0.1% O_2 concentration cultures, neither was their doubling time (about 30h in both conditions) (fig 1b). At later time points, the growth of FDCP-Mix cells cultured at 0.1% O_2 was dramatically reduced, with a doubling time that reached 104h after more than 48h of culture. Since the percentage of apoptotic cells in two conditions was similar (fig 1c) after 24h, 48h and 72h of culture, we concluded that the growth arrest observed at 0.1% O_2 was due to the inhibition of proliferation of FDCP-Mix cells after 24h.

Very low O_2 concentration (0.1%) induces FDCP-Mix cell arrest in G_0

We then analyzed the FDCP-Mix cell cycle status after 24, 48 and 72h of culture at 20 and 0.1% O_2, using double labeling with Propidium Iodide (PI, a quantitative DNA fluorescent dye) and anti Ki-67 antibodies (Ki-67 antigen is expressed in the nucleus of cycling cells and absent from G_0 cells) to discriminate cells in G_0 (DNA =2N; Ki-67$^-$) from cells in G_1 (DNA =2N; Ki-67$^+$), S (2N< DNA <4N; Ki-67$^+$) and G_2/M (DNA =4N; Ki-67$^+$). As shown in fig 2a, the percentage of FDCP-Mix cells in G_0 remained stable (13.6±2.3%) during the 72h of culture at 20% O_2. In contrast, at 0.1% O_2 the percentage of G_0 cells significantly increased to 48.8±5.4% after 72h of culture, while the percentage of cells in G_1, S and G_2M decreased in parallel (fig 2b to d). To determine whether this G_0 phase accumulation was due to cells previously in G_0 and G_1 or to cells entering in G_0 after one or several divisions, FDCP-Mix cells labeled with carboxyfluorescein diacetate succinimidyl diester (CFSE) (a stable vital cellular fluorescent dye whose decay allows to analyze cell divisions) were cultured for 72h at

20% O_2 and at 0.1% O_2. At this time point, division rates (CFSE labeling) and cell cycle status (Ki-67$^+$ or Ki-67$^-$) of cultured FSCP-Mix cells were co-analyzed by flow cytometry (FCM). As demonstrated by the comparison of panels b and c of fig 3, the division rate of FDCP-Mix cells was slower at 0.1% than at 20% O_2. This is due to the association of two cell cycle events: a) the extension of the cell cycle duration as concluded from the respective percentages of Ki-67$^+$ cells in each division class and b) the progressive accumulation of G_0 cells after each division (revealed by comparing the percentages of Ki-67$^-$ cells in each division class at 20 and 0.1% O_2).

Very low O_2 concentration (0.1%) induces the differentiation of a majority of FDCP-Mix cells

May-Grünwald-Giemsa staining of FDCP-Mix cytospins showed that after 72h of culture at 0.1% O_2, the majority of cells acquired a granulo-monocytic differentiation (fig 4a) that was confirmed by FCM analysis of the expression of Gr-1 (granulocytic differentiation marker), Mac-1 (monocytic differentiation marker), as well as CD34 and CD117 (markers of primitive hematopoietic progenitors) cell surface markers (fig 4b, c and d). Indeed, after 3 days of culture at 0.1% O_2, the percentage of cells positive for Gr-1 and CD117 respectively increased from 26 to 63% and decreased from 27 to 14 % (fig 4b and c). In parallel, as expected, we observed a decrease of the Mean Fluorescence Intensity (MFI) of the CD34 primitive marker together with an increase of the MFI of the granulo-monocytic marker Mac-1 and Gr-1 (fig 4d). These results confirmed that culture at 0.1% O_2 induces the granulo-monocytic differentiation of the FDCP-Mix cell line.

Very low O_2 concentration (0.1%) also maintains a minor population of FDCP-Mix cells with culture-repopulating ability (CRA)

FDCP-Mix cells issued from 72h of LC1 at 0.1% O_2 were sequentially replated every 72h at 1.10^5 cells/ml at 20% O_2 to test their ability to repopulate later liquid cultures (LC2 to LC5), as determined by counting the total number of cells in LC2 to LC5 (CRA_{cell}). The growth rate of cells incubated in LC1 at atmospheric O2 concentration remained stable throughout the experiment (left part of the graph fig 5a). The growth rate of cells issued from LC1 incubated at 0.1% O_2 (right part of the graph fig 5a) in non-selective, normoxic LC2 to LC5 (fig 5a), remained stable during LC2 and LC3, increased during LC4 and reached the growth rate exhibited by cells incubated in normoxic LC1 during LC5. In the meantime the percentage of cells in G_0 decreased and the percentage of cells in G_1, S and G_2M started to increase during LC2 to reach control levels at LC4 (data not shown). In order to confirm that this progressive repopulation of LC was due to the maintenance in hypoxia of a limited cell subpopulation, cells rescued from non-selective (normoxic) LC2 to LC5 were cultured in semisolid medium to identify and count, after 11 days, the number of colonies issued from CFC. The percentage of these CRA_{CFC} at the end of 0.1% O_2 LC1 was 14 times lower than after 20% LC1 and progressively returned to similar values in LC4 (fig 5b).

Relationships between G_0 quiescence, Gr-1 expression and CRA_{CFC} cells.

Since the differentiation after 3 days of culture at 0.1% O_2 was accompanied by the G_0 entry of a large percentage of FDCP-Mix cells, we explored the relationship between these two phenomena. As a first step, we sorted Gr-1^+ and Gr-1^- cells after 3 days of LC1 at 20 or 0.1% O_2. The microscopic observation on May-Grünwald-Giemsa cytospins (fig 6a) confirmed that Gr-1^+ cells had a typical morphology of terminally differentiated granulocytes including donut-shaped nuclei (arrows ►) whereas Gr-1- cells kept round nuclei with more basophilic cytoplasm as observed in control culture conditions (fig 4a). At both O_2 concentrations all CRA_{cell}- and CRA_{CFC}–type cells were contained within the Gr-1^- population (fig 6b and c).

Analysis of the cell cycle status of Gr-1$^+$ and Gr-1$^-$ cells showed that CRA-type undifferentiated Gr-1$^-$ cells were mostly cycling, while differentiated Gr-1$^+$ cells without CRA were in G_0 (fig 6d). In addition, a 24 hours treatment of FDCP-mix cells with 5FU (10µg/mL) at the end of LC1 followed by medium renewal and replating in LC2, dramatically decreased CRA_{cell} and CRA_{CFC} similarly in both O_2 conditions (data not shown). Altogether, these data demonstrate that at very low 0.1% O_2 concentration, most FDCP-Mix cells differentiate and enter in the quiescent G_0 status while a minority of undifferentiated cells, those which maintain the self renewal of the cell line, remain in active cell cycle.

FDCP-Mix cells in G_0 overexpress hypophosphorylated pRb and p27^{KIP1} proteins

Due to their role in the regulation of cell cycling, we analyzed the total amount of p27^{KIP1} and pRb proteins and the phosphorylation status of pRb after 24, 48 and 72h of culture at 20% O_2 and 0.1% O_2. As expected, the total quantity of pRb protein remained stable and similar during the 72h culture at 20 and 0.1% O_2 (fig 7a). S phase transition is dependent on pRb phosphorylation on several specific sites. Therefore, we studied this protein phosphorylation status. Indeed, unphosphorylated pRb progressively increased while phosphorylated pRb disappeared during the 72h culture of FDCP-Mix cells at 0.1% O_2 (data not shown). We then analyzed several pRb phosphorylation sites involved in the G_1 to S phase transition. The ser 608, ser 807/811 and thr 821 sites (stably phosphorylated at 20% O_2) became very faint at 0.1% O_2 (fig 7b). In the meantime, the p27^{KIP1} level in FDCP-Mix cells strongly increased during cultures at 0.1% O_2 while it remained stable at 20% O_2 (fig 8a). Up-regulation of the p27^{KIP1} protein by hypoxia in FDCP-Mix cells was partly related to transcriptional mechanisms since p27^{KIP1} mRNA level significantly increased during cultures at 0.1% O_2 (2.76, 3 and 2.4 fold at 24, 48 and 72h respectively) (fig 8b). In order to know if the regulation of the p27^{KIP1} protein was linked to the G_0 quiescence and differentiation of the

FDCP-Mix cell line, we explored its expression in the Gr-1^{-} and Gr-1^{+} populations by flow cytometry. Both the level of p27^{KIP1} fluorescence intensity (fig 8c and d) and the percentages of cells expressing high levels of p27^{KIP1} were different in Gr-1^{-} and Gr-1^{+} cells.

Discussion

In the last ten years, an increasing number of publications were devoted to the physiological low bone marrow O_2 concentrations (from < 0.1% O_2 in sub-endosteal bone marrow niches to 4-5% in vascular areas; compared to 21% in atmospheric air) and to the exploration of their role in the regulation of hematopoiesis (1, 15, 16). We and others showed that varying O_2 concentrations (from 20 to 0.1%) in cultures of hematopoietic stem and progenitor cells modified their quiescence vs. proliferation and self renewal vs. differentiation balances (2, 4-7, 19-23, 31-33). To investigate the molecules and mechanisms involved in these phenomena we used the FDCP-Mix hematopoietic cell line that is considered to share numerous phenotypic and functional properties with bone marrow hematopoietic progenitors. Indeed, FDCP-mix cells have a normal karyotype, are non-leukemic, strictly depend on IL-3 for their survival and proliferation, and differentiate towards different myeloid lineages when cultured with other cytokines (30). In addition, when cultured at 0.1% O_2 cycling FDCP-Mix cells line progressively accumulated in G_0 after one to few divisions.

This accumulation in G_0 was accompanied by a major granulo-monocytic differentiation as shown by sorting Gr-1$^+$ (differentiated) and Gr-1$^-$ (undifferentiated) FDCP-Mix cells after 3 days of culture at 20 and 0.1% O_2. In both O_2 conditions the Gr-1$^-$ FDCP-Mix subset had a high CRA and a high percentage of cycling cells whereas the Gr-1$^+$ differentiated fraction was mostly in G_0 with a quite null CRA. This result suggests a link between cell cycle arrest and differentiation.

Some articles found a differentiating effect of low O_2 concentrations on leukemic cells either from primoculture or from cell lines (6, 23-25) similar to the one observed with FDCP-Mix cells. In contrast, the group of P. Dello Sbarba showed that when the K562 cell line (issued from a blast-crisis chronic myeloid leukemia -an HSC malignancy- patient) was cultured at 0.1% O_2 for 7 days, the number of cells dramatically decreased (by 1 log), most cells

underwent apoptosis while a minor hypoxia-resistant subpopulation repopulated LC2 incubated at 20% O_2, exhibiting full CRA maintenance (8). Using the murine MEL cell line, they obtained similar results, founding, in addition, at 0.1% O_2 a quiescent subset of CRA cells that resisted to 5FU treatment (4). However, in those two articles, cell differentiation was not studied. When the FDCP-Mix cell line was cultured under similar conditions, apoptosis did not increase significantly, most cells underwent proliferation arrest and differentiated, while a small population of slowly-cycling CRA cells (since killed by 24h of 5-FU) persisted after 3 and even 7 days of culture at 0.1% O_2. These differences between FDCP-Mix (and the leukemic cell lines that differentiate in hypoxia) on one hand and K562, MEL and $CD34^+$ cells on the other hand could be due 1) to the fact that FDCP-Mix and the leukemic cell lines that differentiate in hypoxia (6, 23-25) derive from progenitors and not from HSC, 2) to their lower sensitivity to apoptosis than primary $CD34^+$ cells in hypoxic cultures owing to their immortalized status (but this does not apply to the K562 and MEL cell lines) or 3) to an association of both phenomena.

Our previous results showing that primoculture progenitors disappear rapidly during cultures of murine bone marrow or human $CD34^+$ cells at 1% O_2 (21, 27) while leukemic and FDCP-Mix cell lines survive and differentiate strengthen the hypothesis that the maturation of FDCP-Mix cells in hypoxia is linked to their acquired immortalized status. This allows their prolonged survival as differentiated cells in hypoxia, while primoculture progenitors rapidly die in these conditions before reaching maturation.

The permanent cycling of CRA-type and the absence of apoptosis in cultures of FDCP-Mix cells at very low O_2 concentration that contrasts with the apoptosis of a large percentage and the quiescence of a minority of primoculture $CD34^+$ and MEL cells cannot be explained by the immortalized status of FDCP-Mix since MEL is also a permanent (and leukemic) cell line. We suggest that this major functional difference in response to very low O_2 concentrations is

due to the "stem" or "progenitor" cell status of these different cell types. Together, the above-mentioned results with primary $CD34^+$ cells, K562 cells, other leukemic cell lines and our present work imply that regulation by very low O_2 concentrations (<1%) of cycling, differentiation and survival, is depending on the differentiation level of cells (stem cells vs. progenitors) whether normal or leukemic tissue is in question.

These results led us to explore the molecules involved in the cell cycle modifications induced by hypoxia. The G_0 and/or G_1 (undefined since neither Ki-67 nor any other G_0 marker was used) quiescence of fibroblasts and B lymphocytes induced by serum starvation or hypoxia is linked to $p27^{KIP1}$ increase that blocks the Cyclin/Cyclin Dependent Kinase (CDK) activity, thus leading to pRb hypophosphorylation (11, 28, 29, 34-37). Conversely phosphorylations of pRb on multiple ser/thr sites by Cyclin/CDK during the G_1 phase lead to the release of molecules required for the transition to S phase (38). Briefly, the phosphorylation of thr 821 releases histone deacetylase; the phosphorylation of ser 807/811 releases c-abl and the phosphorylation of ser 608 participates in the release of the master transcription factor E2F. These three molecules play a major role in the G_1 to S transition. Hypoxia dramatically reduced the phosphorylation of those 3 pRb sites in FDCP-Mix cells suggesting that dephosphorylated pRb participate to their quiescence. Hypoxia also induced a significant increase of the $p27^{KIP1}$ protein and mRNA in FDCP-Mix cells. Finally, we showed that the quiescent differentiated Gr-1^+ cells expressed higher $p27^{KIP1}$ levels than the Gr-1^- cycling cells, a fraction containing CRA cells. That is most probably responsible of the accumulation of unphophorylated pRb. Our present data that extend personal preliminary results (39) are similar to those very recently published by Eliasson *at al.* (40). Altogether they show that $p27^{KIP1}$ is involved in the regulation of FDCP-Mix cell cycling in hypoxia.

The last important point of our work is that it revealed a minor stem cell-like subpopulation (that repopulates liquid cultures after returning them to 20% O_2) of the FDCP-mix cell line

that kept its self-renewal potential after 3 to 7 days of culture at 0.1% O_2. Similarly to normal stem cells and to cancer cells, the CRA-type FDCP-Mix cells are highly O_2 independent since they survive and proliferate in nearly anoxic conditions (0.1% O_2). Further experiments will explore a) the metabolic peculiarities of the low-energy proliferation mechanism of Gr-1^{-} FDCP-Mix cells, b) the relationship of this mechanism to self-renewal divisions uncoupled to differentiation and c) the roles of HIF-1, p27^{KIP1} and pRb in the regulation of balance between quiescence and differentiation of FDCP-Mix.

Materials and methods

Maintenance of the FDCP-Mix cell line in culture

The FDCP-Mix cells (kindly provided by G. Mouchiroud ; Lyon, France) were routinely replated at $5x10^4$ cells/mL every 3 days in Iscove's modified Dulbecco's medium (IMDM, Invitrogen, Carlsbad, CA, USA) complemented with penicillin (50 units/mL), streptomycin (50 µg/mL), 20% horse serum (HS, Invitrogen), and 10% Wehi conditioned medium (CM) as source of IL-3. All cultures were carried out at 37°C in a humidified 5% CO_2 incubator (Binder, Tuttlingen, Germany). For experiments, cells in exponential growth at day 3 of culture were centrifuged, washed and resuspended in complete medium containing Wehi-CM as required.

Cell proliferation, viability and apoptosis in ambient and low O_2 concentration

FDCP-Mix cells were seeded at $1x10^5$ cells/mL and cultured for 24, 48 and 72h in the same culture medium either at 20% O_2 in a humidified 5% CO_2 incubator or at 0.1% O_2 concentration in an O_2/CO_2 incubator (Xvivo, BioSpherix, Redfield, NY, USA). After culture, viable cells were counted on Malassez Cell with Trypan Blue exclusion. Apoptosis was measured using the Annexin-V-FITC Kit (#PN IM3546, Beckman Coulter, Fullerton, CA, USA) following manufacturer protocol. Samples were analyzed by flow cytometry (FCM) with Becton Dickinson FACSCanto II and FACSDiva™ software (San Jose, CA, USA).

Analysis of cell cycle and divisions by flow cytometry

Cell cycle was analyzed at 24, 48 and 72 hours of culture at 20% O_2 and at 0.1% O_2. In order to distinguish G_0, G_1, S and G_2/M cell cycle phases, cells were double labeled with Propidium Iodide (PI) (quantitative DNA labeling) and Ki-67 antibody (absent only in G_0 phase cells).

Briefly, cells washed in PBS were incubated 30 min at room temperature (RT) in a PFA/saponin solution (0.4% PFA, 10mM HEPES, 0.02% saponin, H_2O). Cells were washed two times in "washing buffer" (5% FBS, 0.05% sodium azide, 55mg/L EDTA, 0.02% saponin) and incubated 30 min at RT with an anti-Ki-67 antibody or isotype (Alexa Fluor® 647 conjugated anti-Ki-67: #558615, Alexa Fluor® 647 conjugated isotype: #557732, BD Pharmingen, San Diego, CA, USA). Washed cells were resuspended in PBS supplemented with PI (20µg/mL) to quantitatively label DNA. Double labeled cells were then analysed by FCM using a Becton Dickinson FACSCanto II and FACSDiva™ software. In order to know if G_0 cells were issued from cells previously in G_0 and G_1 phases or from cells in S, G_2 and M phases that divided before entering in G_0, cells were labeled with carboxyfluorescein diacetate succinimidyl diester (CFSE; a stable vital cellular fluorescent dye whose decay allows to analyze cell divisions; #C34554, Invitrogen) according to manufacturer instructions. Briefly, $1x10^6$ FDCP-Mix cells were incubated in the dark during 15min at 37°C in 1ml of pre-warmed PBS + 1%BSA and 10µM CFSE. Cells were then washed in FDCP-Mix culture medium and incubated 30 min at 37°C in the dark. After a last wash, cells were resuspended in complete culture medium ($3x10^5$ cells/mL) and incubated (37°C, 20% O_2, 5% CO_2) during 24h to eliminate dead cells and use cell populations able to proliferate. Cell aliquots were then cultured either at 20 or 0.1% O_2 for 24, 48 or 72h before being labeled with anti Ki-67 and PI as described and analyzed by FCM.

Cytospin and May-Grünwald/Giemsa staining

$1.5x10^5$ cells resuspended in 50µL of HS were cytospun at 300 rpm during 20min using a cytocentrifuge (Shandon CytoSpin III, GMI, Ramsey, MN, USA) dried and stained with May-Grünwald before microscopical examination at X100 and X600 magnification (AX70, Olympus, Tokyo, Japan).

Analysis of cell surface markers and cell sorting

Cells cultured at 20% O_2 and at 0.1% O_2 were washed with washing buffer, incubated 30min with fluorescein isothiocyanate or phycoerythrin conjugated Isoptype, anti Mac-1, Gr-1, CD34 and CD117 antibodies (respectively, #22225033, #22159113, #22155243, #22150344 and #22151173, Immunotools, Friesoythe, Germany). Washed cells were then analyzed by FCM using a Becton Dickinson FACSCanto II and FACSDiva™ software or sorted according to their Gr-1 expression using a Becton Dickinson FACSAria.

Analysis of $p27^{KIP1}$ expression in Gr-1$^-$ and Gr-1$^+$ cells

Cells were double labeled first with anti-Gr-1 (as described in the previous paragraph) and then with anti-$p27^{KIP1}$ antibody according mostly to the procedure described for cell cycle analysis. Briefly, cells were incubated for 30 min with biotinylated anti-$p27^{KIP1}$ antibody (diluted 1/300, # MS-256-B, Thermo Scientific, Rockford, IL, USA) instead of anti-Ki-67. Cells were then washed twice with washing buffer before a final 20 min incubation with streptavidin-PE (diluted 1/1000, #IM0557, Brea, CA, USA).

Culture-Repopulating Ability (CRA) assay

After 72h of Liquid Culture (LC) at 20% or 0.1% O_2 (LC1), cells were centrifuged 5 min at 300g at RT and transferred at $1x10^5$ cells/mL in fresh medium into secondary cultures always incubated at 20% O_2, irrespective of whether LC1 had been incubated at 20% or 0.1% O_2 (non-selective LC2). Cells were then further replated every 3 days and always incubated at 20% O_2 (LC3 to LC5). At the end of each LC, cell aliquots were harvested for counting following Trypan Blue exclusion (CRA_{cell}) and CFA assay (CRA_{CFC}).

The CRA assay estimates the maintenance of the stem cell subset contained within a cell population which has undergone a treatment (for instance, incubation in hypoxia) in a selective LC1 by means of cell transfer into non-selective (for instance, normoxic) LC2 and a further incubation therein. LC2 repopulation is then measured by counting the total number of cells (CRA_{cell}) or the number of clonogenic (CFC) cells (CRA_{CFC}) by means of a CFA assay (see below). In normal hematopoietic cell populations, the standard CRA assay has been shown to detect short-term-repopulating hematopoietic stem cells (41).

Colony-Forming Ability (CFA) Assay

$1x10^3$ FDCP-Mix cells were seeded in 250µL of methyl-cellulose (Methocult^{TM}, #H4230, Stemcell technologies, Vancouver, Canada) with 10ng/mL of mIL3 (#213-13, Peprotech, Rocky Hill, NJ, USA) in duplicate. Culture dishes were incubated for 11 days at 37°C in humidified atmosphere with 20% O_2, 5% CO_2 before counting the number of colonies (aggregates >50 cells).

Western Blot Analysis

Cells cultured at 20 % O_2 and at 0.1% O_2 were washed in PBS and lysed in loading buffer (2% SDS, 10% glycerol, 50mM Tris pH7.8). Protein concentration was determined using the BCA Pierce assay (Thermo Scientific). Then, 40µg of whole protein cell extract were resolved in SDS-PAGE and transferred onto a PVDF Membrane (#RPN303F, GE Healthcare, Uppsala, Sweden) to probe for pRb and p27 proteins respectively. The membrane was saturated with non fat milk (5%) in TBS-T (10mM Tris pH 7.8, 150mM NaCl, Tween 0.1%) and then incubated overnight at 4°C with specific antibodies to phospho-ser 608 pRb, phospho-ser 807/811 pRb, $p27^{KIP1}$ (respectively #2181, #9308 and #2552, Cell Signaling Technology, Beverly, MA, USA), pRb (#554136, BD Pharmingen) or phospho-thr 821 pRb

(#44-582, Invitrogen) diluted as indicated by the manufacturer. After 3 washes in TBS-T, membranes were incubated 1 h with antirabbit or antimouse horseradish peroxydase-conjugated antibodies. The bands were visualized using the ECL system (#WBKLS, Millipore, Billerica, MA, USA). The membrane was washed again, saturated, stained with an anti-β-actin antibody (#A5316, Sigma, St Louis, MO, USA) and finally revealed as described above.

Reverse transcription and quantitative PCR

Cells cultured at 20 % O_2 and at 0.1% O_2 were washed in PBS and treated with 250µL Trizol (#15596-026, Invitrogen). Total RNA extraction, DNAse treatment (#M6101, Promega, Madison, WI, USA) and reverse transcription with M-MLV Reverse Transcriptase (#M1705, Promega) were realized according to manufacturer's instructions. Samples were run three times in duplicates using the brilliant SYBR Green QPCR (#600548, Stratagene, La Jolla, Ca, USA) in the MX4000 apparatus (Stratagene) according to the manufacturer's instruction. The real-time PCR reactions were carried out in the presence of mouse $p27^{KIP1}$ gene specific oligonucleotide primers (forward primer: 5'AGGCGGTGCCTTTAATTGGG, reverse primer: 5'TTACGTCTGGCGTCGAAGGC) or 36B4 gene specific oligonucleotide primers (forward primer: 5'-GCTTCATTGTGGGAGCAGAC and reverse primer: 5'CATGGTGTTCTTGCCCATCAG): 10 min activation at 95°C, followed by 40 cycles at 95°C for 30 sec, 58°C for 1 min and 72°C for 1 min.

A relative quantification of $p27^{KIP1}$ transcript expression was obtained by standardizing to 36B4 levels that was shown to be stable in hypoxia (42). The final value was calculated by taking an average of relative transcript copy numbers amplified from the 3 sets of PCR reaction performed in duplicates.

Statistics

All data are presented as mean ± standard deviation of 3 to 7 independents experiments. Friedman or Kruskal & Wallis test were respectively used to compare more than 2 experimental conditions paired or not. For statistical analysis of differences between 2 not paired experimental conditions, Mann & Whitney test were used.

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Aknowledgements

This work was funded by grants from la ligue nationale contre le cancer (comité sud-ouest) and association Laurette Fugain. AG, CD and FH received a doctoral fellowship from the French ministère de l'Education Nationale, de la Recherche et de la Technologie.

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The language corrections of manuscript by Mrs. Elisabeth Volkmann are highly appreciated.

Titles and legends to figures

Figure 1: Influence of O_2 concentration on the proliferation and apoptosis of FDCP-Mix cells. Cells were seeded at $1x10^5$ cells/mL and cultured 24, 48 and 72h at 20, 3, 1 or 0.1% O_2.

a) Living cells count.

b) The cell doubling time was calculated as follows: *t*ln2/ln (cf/ci)*. *t* : time of culture in hours; *ln* : natural logarithm; *cf* and *ci* : living cells concentrations at final and initial culture time points respectively.

c) Percentage of apoptotic cells (annexin V positive cells).

Values are the mean ± standard deviation of 5 independent experiments [†$p<0.05$; Kruskal & Wallis test].

Figure 2: Influence of O_2 concentration on the cell cycle of FDCP-Mix cells. Cells were seeded at $1x10^5$ cells/mL and cultured at 20% or 0.1% O_2 for 24h, 48h and 72h. Double labeling with PI and Ki-67 antibody identifies cells (a) in G_0 (DNA =2N; Ki-67^-), (b) in G_1 (DNA =2N; Ki-67^+), (c) in S (2N< DNA <4N; Ki-67^+) and (d) in G_2/M (DNA =4N; Ki-67^+). Values are the mean ± standard deviation of 7 independent experiments. [†$p<0.05$ *$p<0.01$; Friedman test]

Figure 3: Hypoxia (0.1% O_2) reduces the number of cell divisions of FDCP-Mix cells. Dot Plots of FDCP-Mix cells labeled with CFSE and anti Ki-67 antibody. Cells before culture (a) and after 72h of culture at 20% (b) and 0.1% (c) O_2. The Ki-67 antibody labeling discriminates quiescent cells (Ki-67^-) from cycling cells (Ki-67^+). Decreasing levels of CFSE fluorescence (from right to left) reflects the increasing number of cell divisions. One representative experiment out of 3.

Figure 4: Hypoxia (0.1% O_2) increases granulo-monocytic differentiation of FDCP-mix cells.

a) After 72h of culture at 20% and 0.1% O_2 cells were harvested, cytospun and stained with May-Grünwald Giemsa.

b) After 72h of culture at 20% and 0.1% O_2 cells were labeled with appropriate isotypic antibodies, anti-Gr-1, anti-Mac-1, anti-CD34 or anti-CD117 specific antibodies and analysed by FCM. Histograms of one representative experiment are shown.

c) This table gives the mean ± standard deviation of the percentage of cells positive for the Gr-1, Mac-1, CD34 and CD117 markers after 3 days of culture at 20% and 0.1% O_2. These percentages were calculated from 3 independent experiments.

d) Variation of the MFI of Gr-1, Mac-1, CD34 and CD117 positive cells after 72h of culture at 20% or 0.1% O2. For each marker, the value represents the mean ratio ± standard deviation of the MFI measured at 0.1% O_2 / MFI measured at 20% O_2 in 3 independent experiments.

Figure 5: Hypoxia (0.1%) maintains a minor population of FDCP-Mix cells with Culture Repopulating Ability (CRA).

After 3 days of primary Liquid Culture (LC1) at 20% or 0.1% O_2 cells were serially replated at $1x10^5$ cells/ml at 20% O_2 every 3 days (LC2 to LC5). At the end of each LC (LC1 to LC5), a) the number of living cells (CRA_{cell}) in liquid culture was counted and b) their CFC content were revealed by the CFA assay that allowed to count the number of colonies after 11 days of culture at 20% in semisolid medium (CRA_{CFC}).

Values are the mean ± standard deviation from 4 independent experiments. Statistical analysis of differences between similar LC at 20% and at 0.1% O_2 was performed with Mann & Whitney test [†$p<0.05$, *$p<0.01$]. Statistical analysis of differences between serial LCs in the 20% and 0.1% O_2 group used the Friedman test [†$p<0.05$].

Figure 6: Gr-1$^+$ and Gr-1$^-$ FDCP-Mix cells have different morphology, CRA_{cell} and CRA_{CFC} capacities and cell cycle status. After 3 days of LC1 at 20% or 0.1% O_2 cells were sorted according to their absence or presence of the Gr-1 antigen.

a) Gr-1$^-$ and Gr-1$^+$ cells issued from 20% and 0.1% O_2 LC1 were cytospun and stained with May-Grünwald-Giemsa and observed at 600X magnification. Arrows (►) indicate cells with donut-shaped nuclei characteristic of terminally differentiated murine granulocytes.

b) Gr-1$^-$ and Gr-1$^+$ cells issued from 20% and 0.1% O_2 LC1 were serially replated at 20% O_2 every 3 days (LC2 and LC3) and living cells were counted.

c) Gr-1$^-$ and Gr-1$^+$ cells issued from 20% and 0.1% O_2 LC1 were plated in semisolid culture to measure their CFA.

d) Distribution of the percentage of G_0 cells in the Gr-1$^-$ and Gr-1$^+$ cell subpopulations issued from 20% and 0.1% O_2 LC1.

Values are the mean ± standard deviation of 3 independent experiments. [*$p<0.001$; Mann & Whitney].

Figure 7: Time dependent modifications of pRb during culture in hypoxia. After 24, 48 or 72h of culture at 20% O_2 or 0.1% O_2, FDCP-Mix cell lysates were analysed by western blot.

a) Time- and O_2 concentration- dependant expression of pRb,

b) Time- and O_2 concentration- dependant expression of phospho-ser 608 pRb, phospho-ser 807/811 pRb, phospho-thr 821 pRb.

Detection of β-actin was used as loading control.

Figure 8: Increase of $p27^{KIP1}$ during culture of FDCP-Mix cells at 0.1% O_2 is related to their terminal granulocytic differentiation.

a) Time- and O_2 concentration- dependant expression of $p27^{KIP1}$ was analysed by western blot.

Detection of β-actin was used as loading control.

b) $p27^{KIP1}$ mRNA level (arbitrary units) was quantified by RT-qPCR after 24, 48 and 72h of culture at 20% O_2 or at 0.1% O_2.

Results are the mean ± standard deviation of 4 independent experiments performed in duplicates. [**$p< 0.005$, *$p< 0.05$; Mann & Whitney test].

c) Flow cytometric analysis of the $p27^{KIP1}$ expression in Gr-1^- and Gr-1^+ populations. FDCP-Mix cells were double labeled (anti-Gr-1 and anti-$p27^{KIP1}$ antibodies) after 3 days of culture at 20% and 0.1% O_2.

d) Percentages of Gr-1^- and Gr-1^+ cells with high and low $p27^{KIP1}$ expression after 3 days of culture at 20% and 0.1% O_2. High and low $p27^{KIP1}$ cells were defined as indicated on the flow cytometry histogram (left panel). The right panel graph shows the mean percentage ± standard deviation of 4 independent experiments.

Figure 1

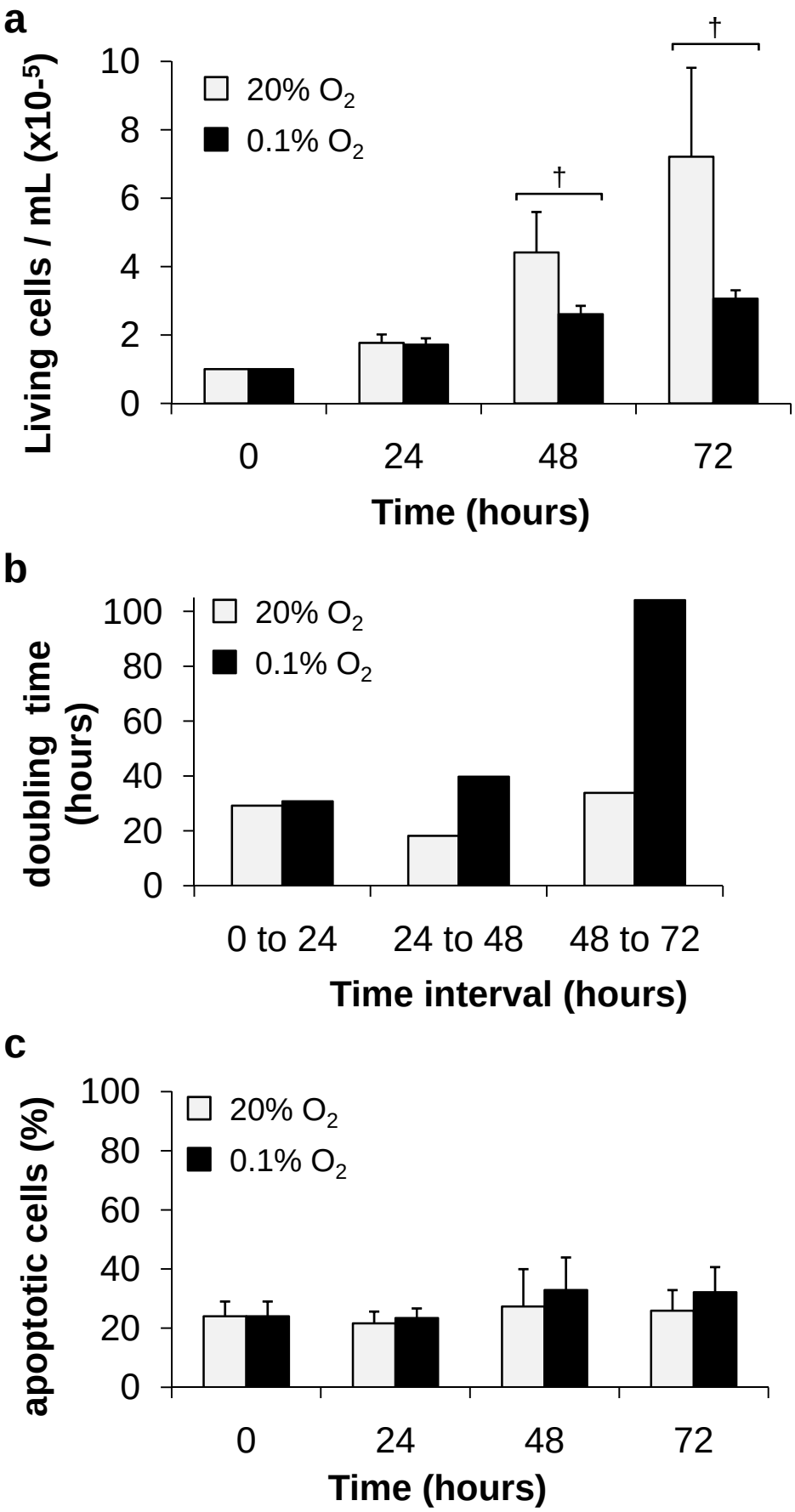

a
Living cells / mL (x10-5)
10
8
6
4
2
0
20% O2
0.1% O2
†
†
0
24
48
72
Time (hours)
b
doubling time (hours)
100
80
60
40
20
0
20% O2
0.1% O2
0 to 24
24 to 48
48 to 72
Time interval (hours)
c
apoptotic cells (%)
100
80
60
40
20
0
20% O2
0.1% O2
0
24
48
72
Time (hours)

Figure 2

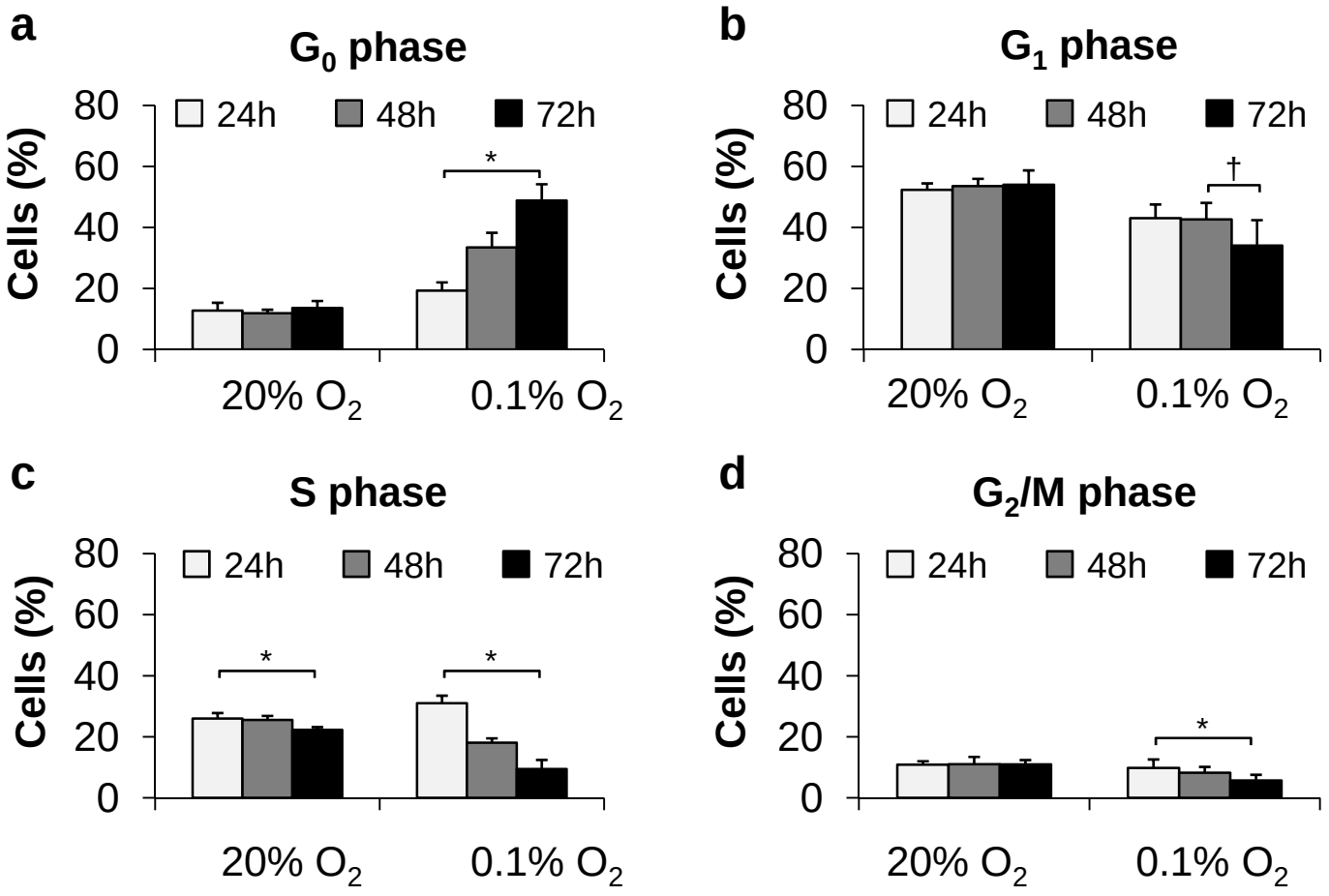

a
G0 phase
Cells (%)
80
60
40
20
0
24h
48h
72h
*
20% O2
0.1% O2
b
G1 phase
Cells (%)
80
60
40
20
0
24h
48h
72h
†
20% O2
0.1% O2
c
S phase
Cells (%)
80
60
40
20
0
24h
48h
72h
*
*
20% O2
0.1% O2
d
G2/M phase
Cells (%)
80
60
40
20
0
24h
48h
72h
*
20% O2
0.1% O2

Figure 3

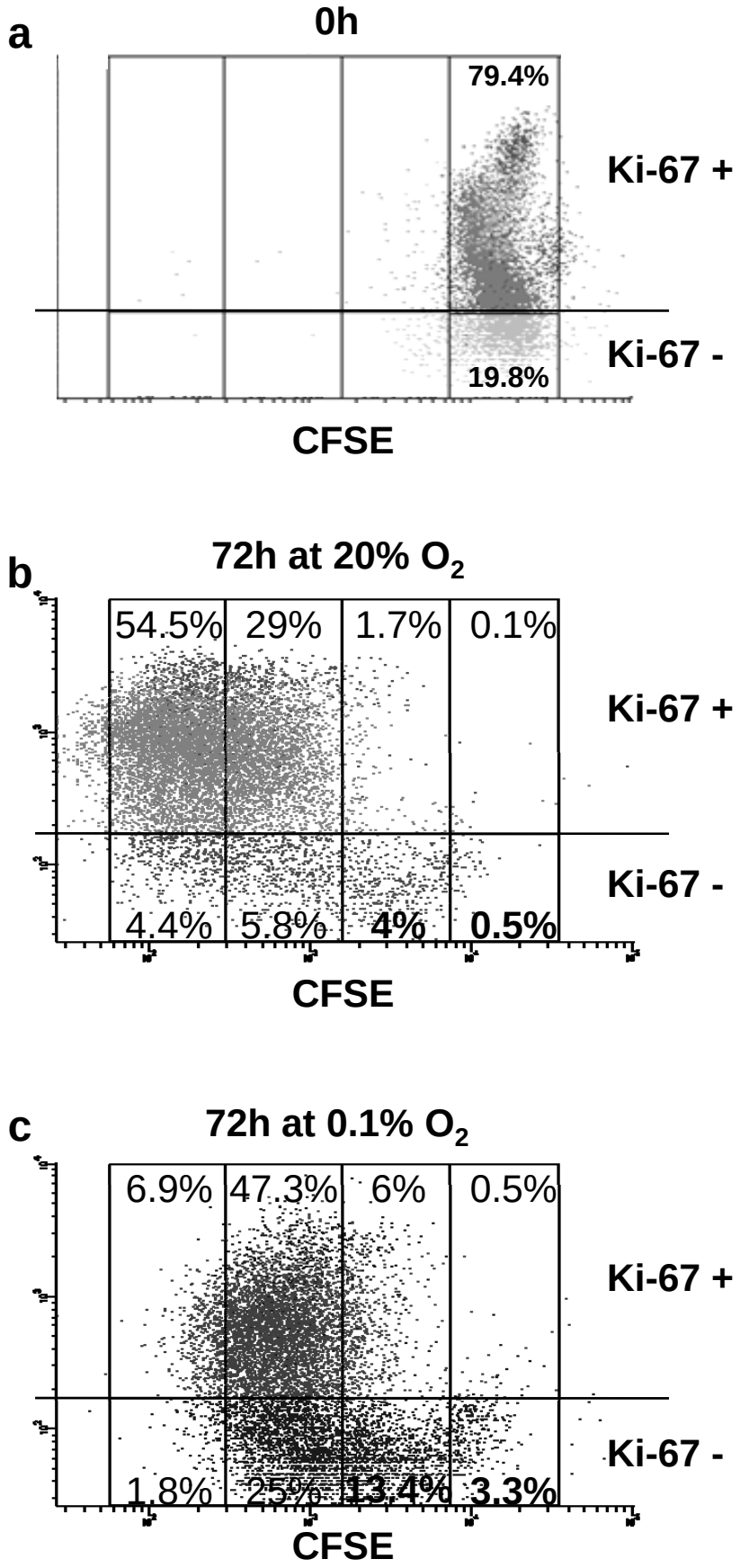
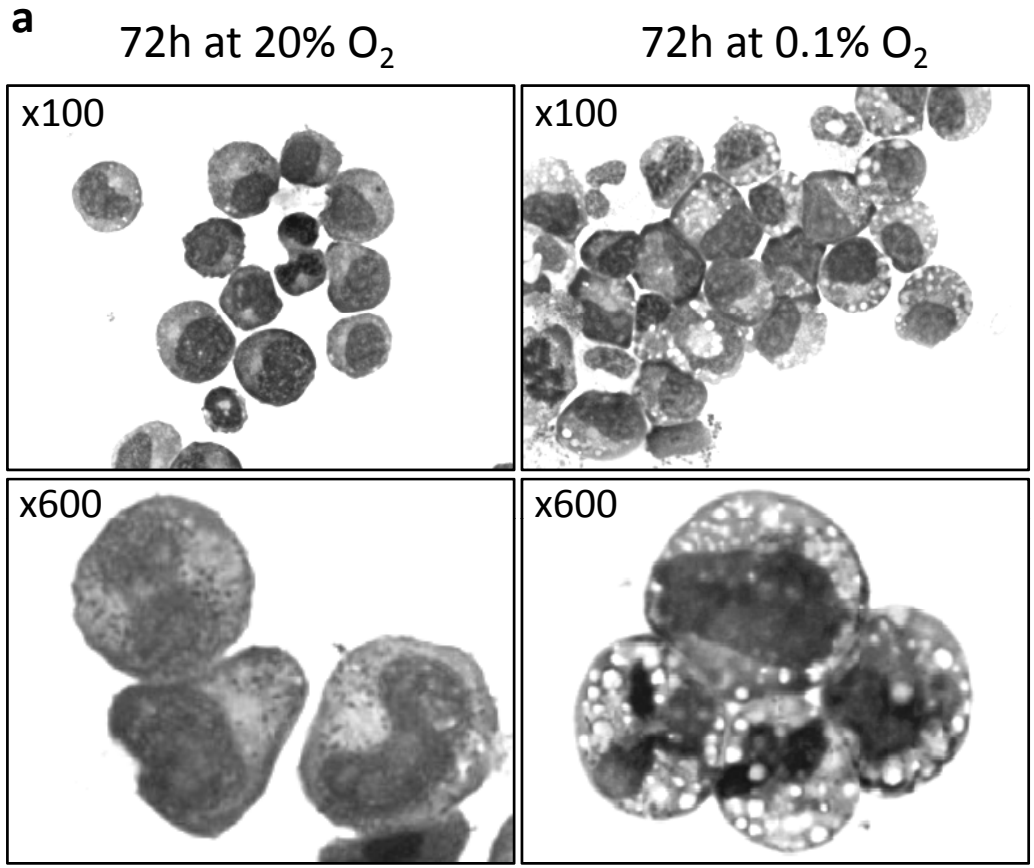
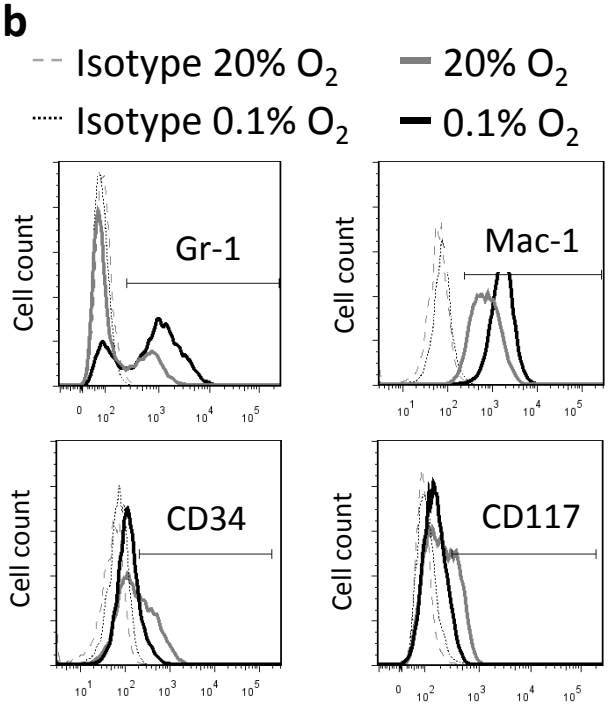

a
0h
79.4%
Ki-67 +
Ki-67 -
19.8%
CFSE
b
72h at 20% O2
54.5%
29%
1.7%
0.1%
Ki-67 +
Ki-67 -
4.4%
5.8%
4%
0.5%
CFSE
c
72h at 0.1% O2
6.9%
47.3%
6%
0.5%
Ki-67 +
Ki-67 -
1.8%
25%
13.4%
3.3%
CFSE

Figure 4

a



b



c

	20% O_2	0.1% O_2
Gr-1	26,1% ± 4,06	63,7% ± 10,61
Mac-1	95,8% ± 2,89	98,6% ± 1,1
CD34	38,9% ± 7,65	18,2% ± 6,96
CD117	26,9% ± 4,26	13,5% ± 6,82

d

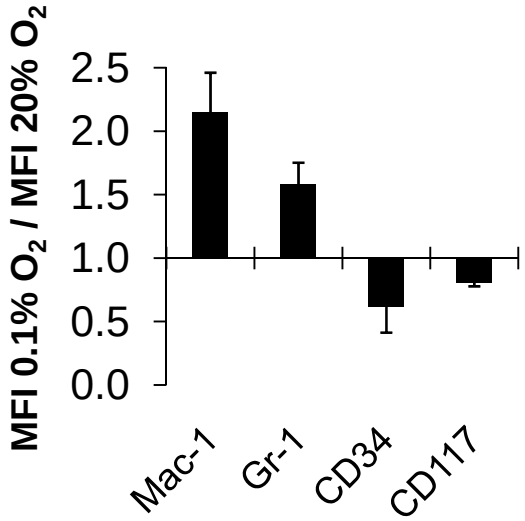


Figure 5

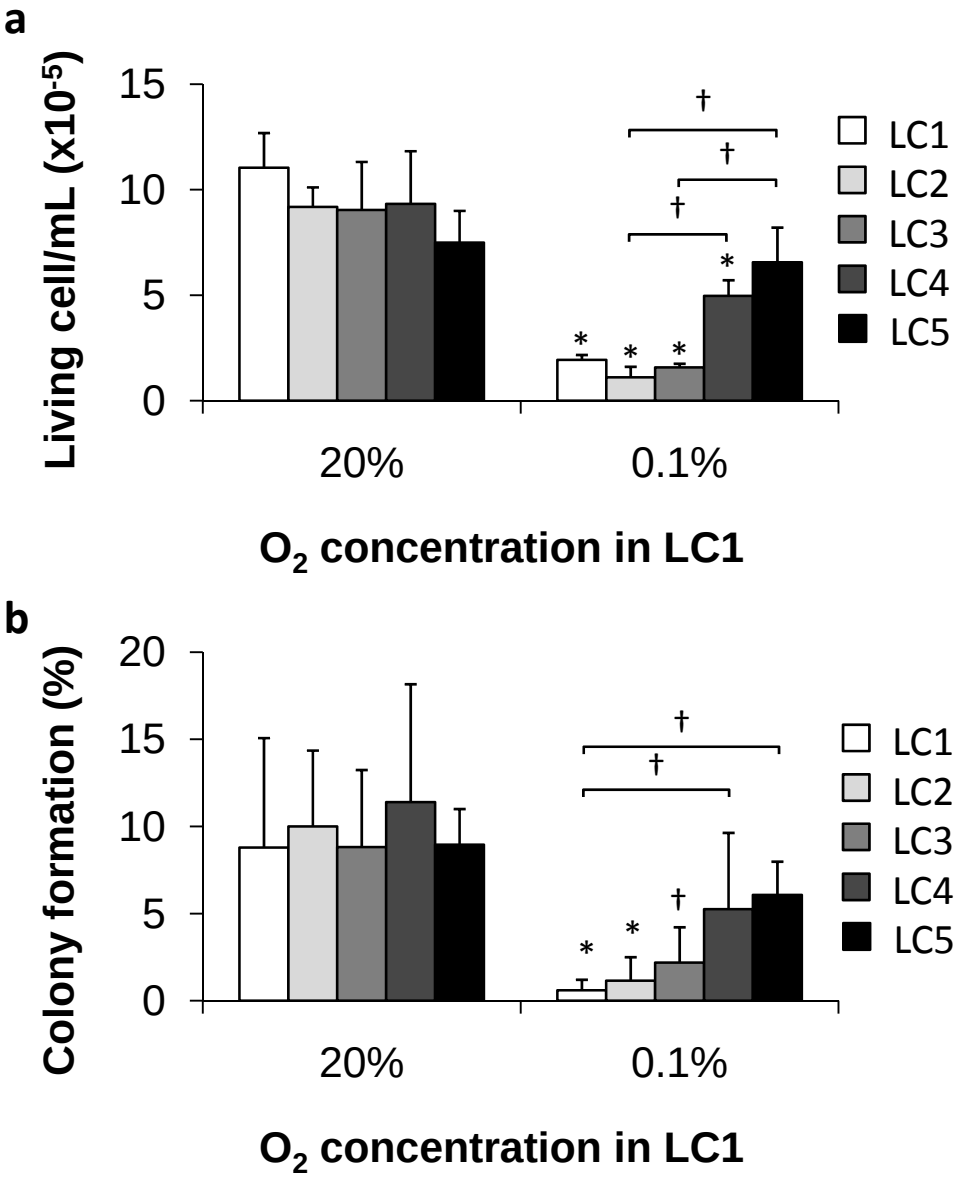

a
Living cell/mL (x10-5)
15
10
5
0
†
†
†
*
*
*
*
LC1
LC2
LC3
LC4
LC5
20%
0.1%
O2 concentration in LC1
b
Colony formation (%)
20
15
10
5
0
†
†
†
*
*
LC1
LC2
LC3
LC4
LC5
20%
0.1%
O2 concentration in LC1

Figure 6

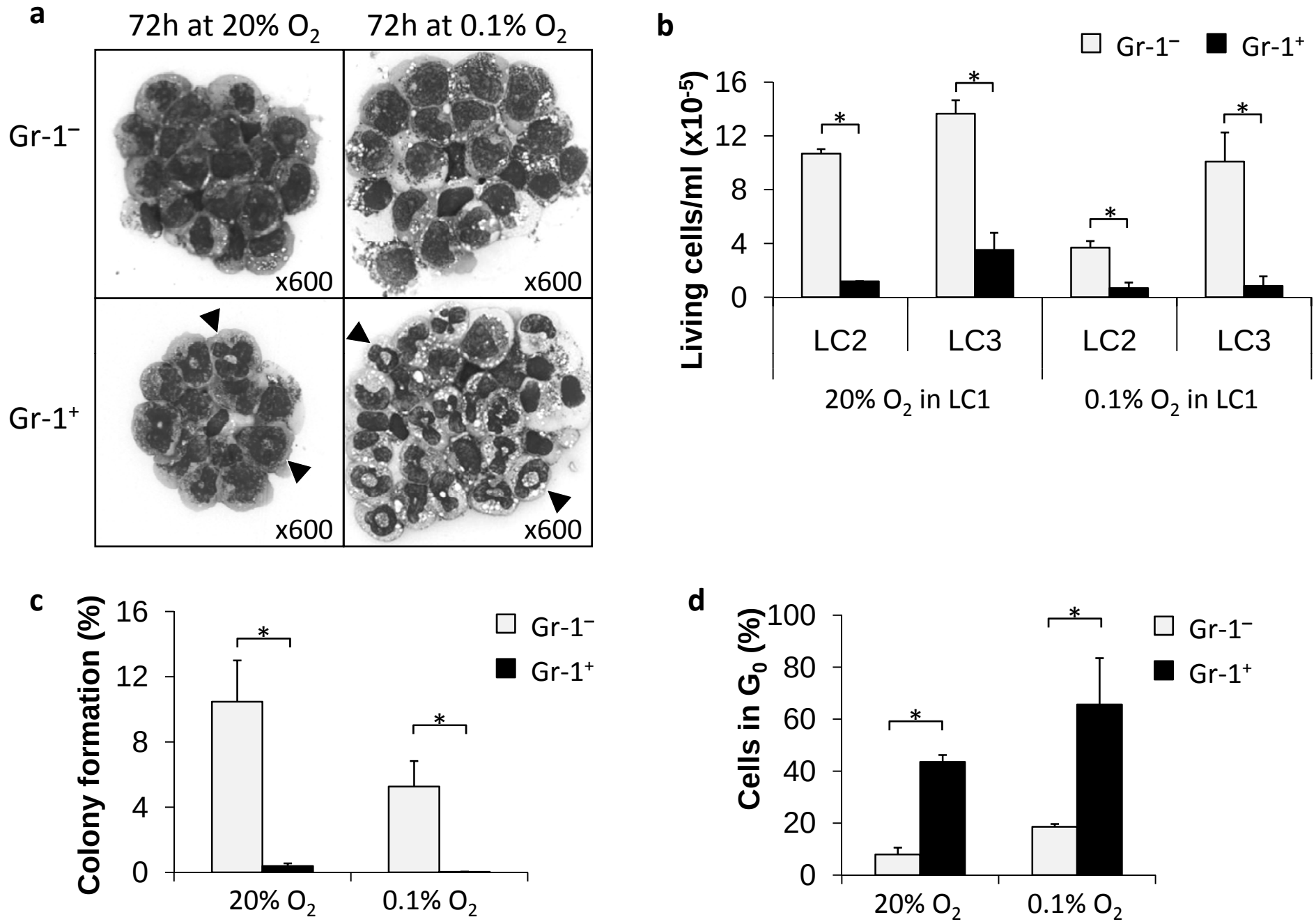

a
72h at 20% O2
72h at 0.1% O2
Gr-1⁻
Gr-1⁺
x600
x600
x600
x600
b
Gr-1⁻
Gr-1⁺
Living cells/ml (x10⁻⁵)
16
12
8
4
0
*
LC2
LC3
LC2
LC3
20% O2 in LC1
0.1% O2 in LC1
c
Colony formation (%)
16
12
8
4
0
20% O2
0.1% O2
Gr-1⁻
Gr-1⁺
d
Cells in G0 (%)
100
80
60
40
20
0
20% O2
0.1% O2
Gr-1⁻
Gr-1⁺

Figure 7

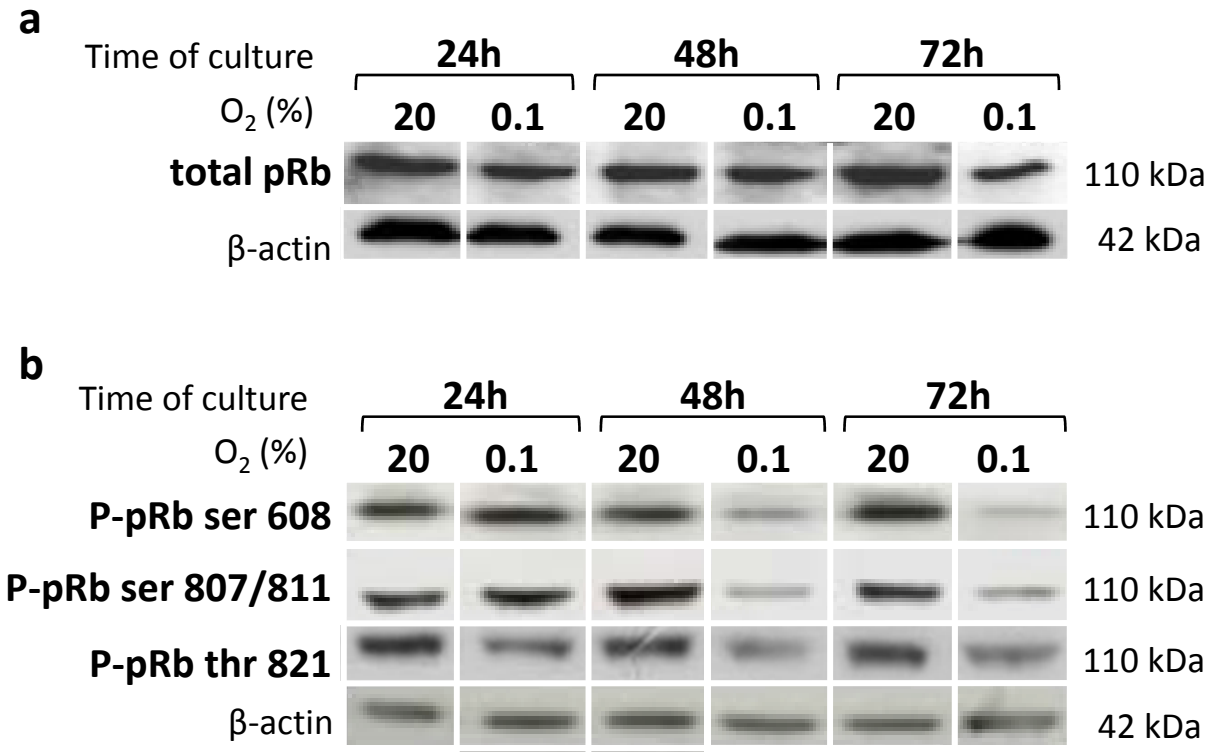
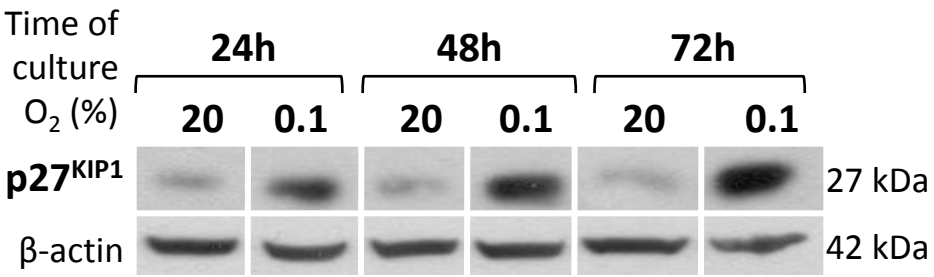
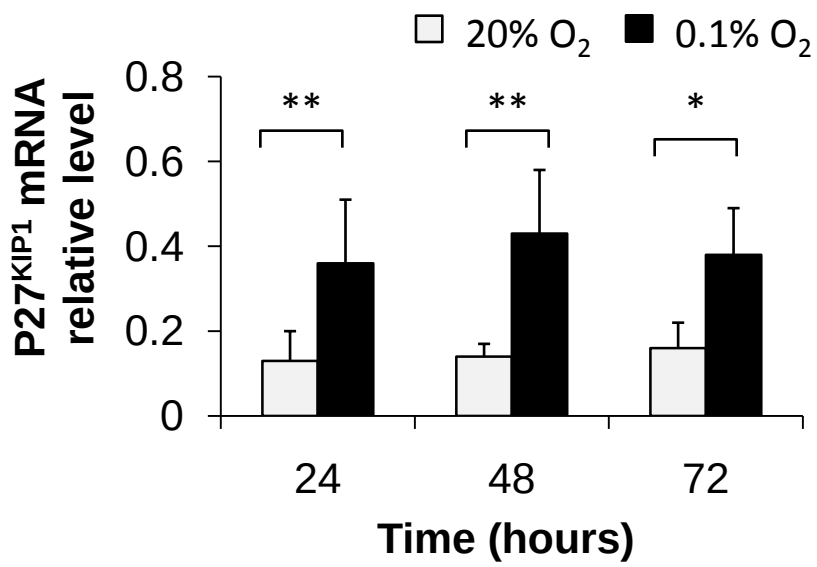

a
Time of culture
24h
48h
72h
O2 (%)
20
0.1
20
0.1
20
0.1
total pRb
110 kDa
β-actin
42 kDa
b
Time of culture
24h
48h
72h
O2 (%)
20
0.1
20
0.1
20
0.1
P-pRb ser 608
110 kDa
P-pRb ser 807/811
110 kDa
P-pRb thr 821
110 kDa
β-actin
42 kDa

Figure 8

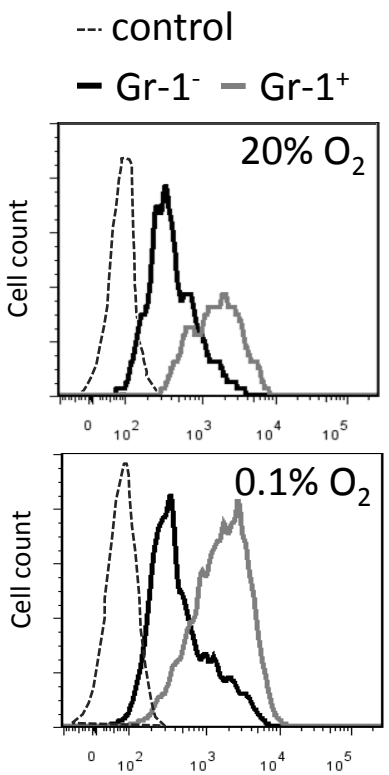
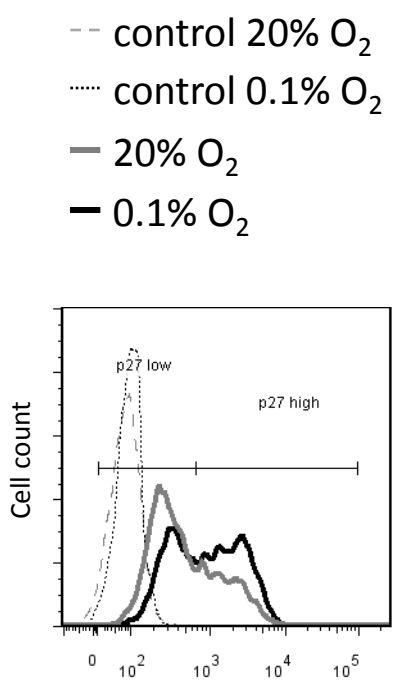
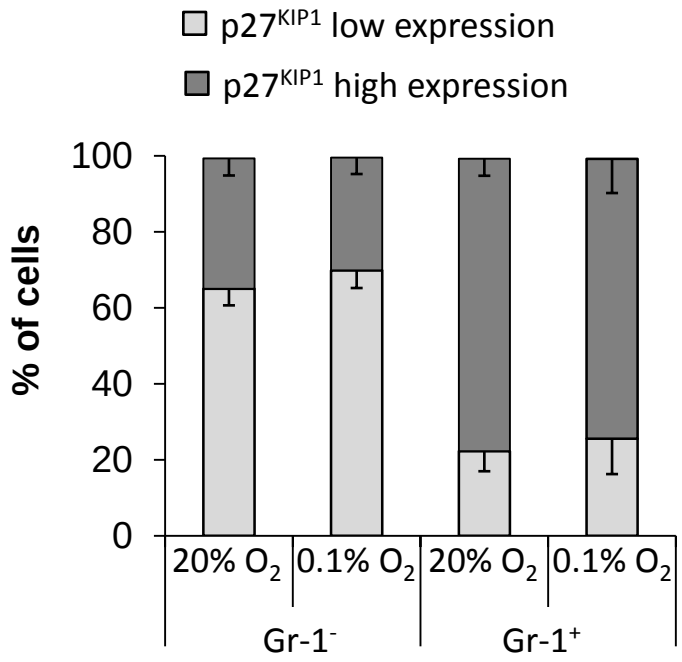
a



b



c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