

Oxygen tension modulates the expression of cytokine receptors, transcription factors, and lineage-specific markers in cultured human megakaryocytes

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Objective. We have recently reported that 20% O₂ significantly enhances total megakaryocyte (Mk) number, polyploidy, and proplatelet formation compared to 5% O₂ in culture. In order to further elucidate the regulatory role of pO₂ on megakaryocytopoiesis, we conducted a kinetic study of the expression of surface markers CD41a and CD42a; receptors for thrombopoietin (TPO), interleukin-3 (IL-3), and Flt3-ligand; the glutamate receptor of the N-methyl-D-aspartate subtype 1 (NMDAR1); and transcription factors GATA-1, NF-E2, and E2F-1.

Materials and Methods. Mks were generated from mobilized peripheral blood (PB) CD34⁺ cells from normal donors in serum-free medium with TPO, IL-3, and Flt3-ligand at 20% and 5% O₂. Quantitative assessment of Mk surface receptors and nuclear transcription factors was performed using multiparameter flow cytometry. mRNA levels of the nuclear transcription factors GATA-1 and NF-E2 were evaluated using RT-PCR.

Results. The proportions of cells expressing the early Mk marker CD41a and the late Mk marker CD42a at day 15 were 4 and 5 times higher, respectively, at 20% O₂. CD41a and CD42a protein levels per cell were also higher at 20% O₂. After day 5, c-Mpl (TPO receptor) generally followed similar kinetics as CD41a. The proportion of IL-3 receptor (IL-3R)⁺⁺ Mks at day 5 was 1.5 times higher at 5% O₂. The NMDAR1 protein previously known to be expressed by neuronal cells has recently been identified in Mks. NMDAR1 and the transcription factors were studied on days 6, 9, and 11. NMDAR1 was expressed at a 1.5- to 1.8-fold higher level at 5% O₂. Twenty percent O₂ supported higher expression of the Mk-early and -late-maturation-specific transcription factors GATA-1 (1.2- to 2.2-fold higher) and NF-E2 (1.1- to 2.8-fold higher). This was consistent with RT-PCR data indicating the presence of higher levels of GATA-1 and NF-E2 mRNA at 20% O₂. E2F-1, a ubiquitously expressed cell cycle transcription factor, was expressed at a 1.5-fold higher level at 20% O₂ on day 6, but this difference did not persist by day 9.

Conclusion. These findings demonstrate that cytokine receptors c-Mpl and IL-3R, and Mk differentiation-specific surface receptors CD41a, CD42a, and NMDAR1, are significantly modulated by pO₂, and suggest that one of the mechanisms of enhanced maturation at 20% O₂ may involve regulation of transcription factors GATA-1 and NF-E2. © 2001 International Society for Experimental Hematology. Published by Elsevier Science Inc.

Megakaryocytes (Mks) mature adjacent to bone marrow (BM) sinus walls and subsequently release platelets within the sinusoidal space or in lung capillaries [1–5]. These sites of platelet production have higher levels of pO₂ compared to the core of the BM where stem and progenitor cells reside

[6–8]. We have recently reported that oxygen tension (pO₂) has profound effects on human Mk maturation [9]. At day 15, mobilized peripheral blood (PB) CD34⁺ cell cultures with thrombopoietin (TPO), interleukin-3 (IL-3), and Flt3-ligand had 2.6-fold more CD41a⁺ Mks, eightfold more Mks with high ploidy (8N or greater), and fivefold more Mks with proplatelets at 20% O₂ vs 5% O₂. Cultures initiated with CD41a⁺ Mks showed similar pO₂ effects, indicating that pO₂ directly affects Mks.

In an effort to unveil the molecular basis for the pheno-

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typic effects of pO_2 on Mks, we conducted detailed kinetic expression-level studies of several Mk-related glycoproteins. Mk-specific markers CD41a (GPIIb/IIIa), which is up-regulated early during megakaryocytopoiesis, and CD42a (GPIX), which is up-regulated relatively later [10–12], were studied in terms of the fraction of positive cells, as well as the levels of these proteins expressed on a per cell basis, via flow cytometry.

Considering the essential role of growth factors in hematopoietic cell survival, proliferation, and differentiation, we examined the kinetics of receptor expression for the growth factors TPO, IL-3, and Flt3-ligand. Since growth factors are provided in saturating concentrations in these cultures, the number of occupied receptors at steady state is likely to be proportional to the number of receptors present on the cell surface. For the IL-3 receptor (IL-3R) and Flt3, cells were stained simultaneously with antibodies against CD41a. This allowed us to determine whether any pO_2 -regulated changes in receptor expression were Mk-lineage-specific.

Glutamate receptors of the N-methyl-D-aspartate (NMDA) subtype are responsible for synaptic neurotransmission in the central nervous system [13,14]. In stroke, a decrease in O_2 supply (hypoxia) leads to release of excess glutamate, which in turn hyperactivates the NMDA receptors of postsynaptic cells [13]. Recently, it was discovered that Mks express NMDA receptor subtype 1 (NMDAR1), and that the NMDAR may play a role in the phorbol myristate acetate (PMA)-induced differentiation of the MEG-01 Mk cell line [15].

Lineage-specific phenotypes, including cytokine receptor expression, are often regulated (in part) by lineage-restricted transcription factors [16,17]. GATA-1 and NF-E2 are transcription factors restricted to selected blood cell lineages including the Mk and erythroid lineages. Multiple GATA-1 binding sites are present in genes encoding Mk-related proteins such as c-Mpl [18], GPIIb [19], GPIX [20], GPIIb α [21], GPIIb β [22], and platelet factor 4 (PF4) [23]. Forced expression of GATA-1 in the murine myeloid leukemic cell line M1 resulted in elevated levels of c-Mpl and PF4 mRNA [24]. GATA-1-deficient mice are thrombocytopenic and their Mks are small, and a significant fraction of these Mks do not undergo endomitosis [25,26]. These cells also contain lower mRNA levels for GPIIb α , GPIIb β , PF4, c-Mpl, and p45 NF-E2 [26]. NF-E2 is an obligate heterodimer of a 45-kDa hematopoietic-tissue-restricted polypeptide known as p45 and a widely expressed 18-kDa small Maf protein [17]. p45 NF-E2 $^{-/-}$ mice show profound thrombocytopenia due to a block in Mk late cytoplasmic maturation. Mks in this case are polyploid, but show significantly fewer granules, disorganized demarcation membranes, and an absence of platelet territories. These Mks are also unable to produce proplatelets in culture [27]. Based on data from GATA-1 and NF-E2 knockout mice, GATA-1 appears to be involved in the transition of immature Mks to mature Mks, whereas NF-E2 appears to play an essential role in terminal Mk maturation and platelet release [26]. Both GATA-1 and NF-

E2-deficient mice exhibit hyperproliferation of Mks [25,26,28]. However, in fetal liver cell cultures from these transgenic mice, only GATA-1-deficient Mk progenitors show enhanced proliferation, whereas colony-forming unit-Mks (CFU-Mks) lacking NF-E2 show substantially lower proliferation potential compared to the wild type [26,28].

E2F-1 is a ubiquitously expressed transcription factor that regulates cell cycle progression by activating genes involved in cell division [29]. Expression of E2F-1 is low in G0 and maximal at the G1/S cell cycle transition. Forced expression of E2F-1 under the control of the PF4 promoter results in dysregulated proliferation of Mks in transgenic mice [30]. Also, these Mks have maturational abnormalities such as hyperdemarcation of cytoplasmic membranes and fewer α granules, and they are incapable of producing platelets. It has been suggested that excessive cell-cycle stimulation by forced E2F-1 expression leads to a block in Mk terminal maturation [30].

The transcription factors GATA-1, NF-E2, and E2F-1 were studied by flow cytometry, which allowed us to double stain the cultured cells for CD41a. For GATA-1 and NF-E2, RT-PCR was used to confirm the flow cytometry results.

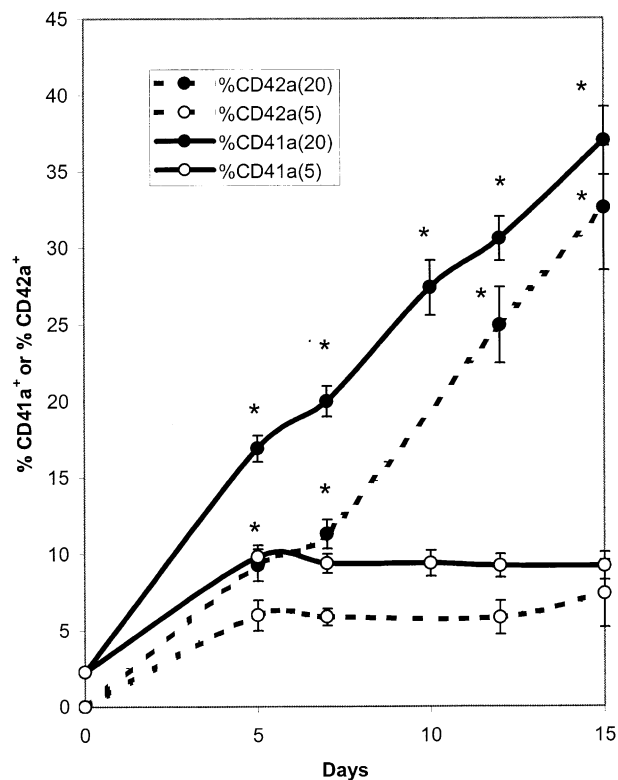


Figure 1. The percentages of CD41a $^{+}$ and CD42a $^{+}$ cells in culture are higher at 20% O_2 ($n = 4$ for days 5–12; $n = 3$ for day 15). Cultures were initiated with CD34 $^{+}$ cells selected from apheresis products obtained from normal donors mobilized with G-CSF, and were carried out in serum-free medium supplemented with TPO, IL-3, and Flt-3 ligand, under an atmosphere containing either 20% or 5% O_2 . * indicates a significant difference ($p < 0.05$) between 20% and 5% O_2 cultures.

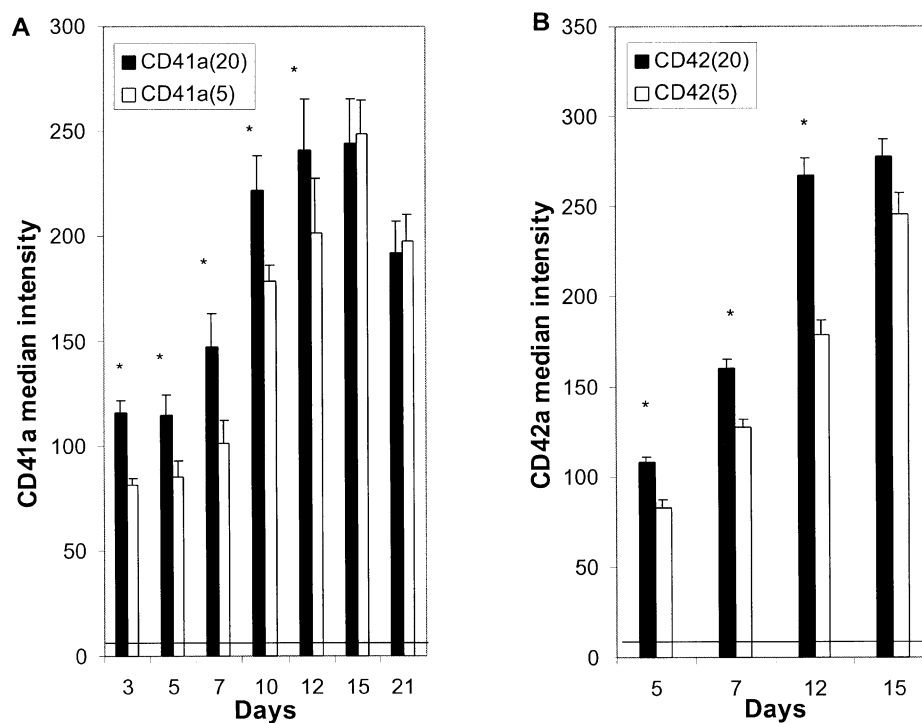


Figure 2. (A) The CD41a protein is expressed at a higher level at 20% O₂ (n = 24). (B) The CD42a protein is expressed at a higher level at 20% O₂ (n = 4 for days 5–12; n = 3 for day 15). Culture conditions are described in the legend to Figure 1. The horizontal line indicates the median fluorescence intensity of cells incubated with nonspecific antibody. * indicates a significant difference ($p < 0.05$) between 20% and 5% O₂ cultures.

Materials and methods

CD34⁺ cell cultures

Cultures were initiated with adult human CD34⁺ cells selected from apheresis products obtained from normal donors mobilized with G-CSF (Poietics Technologies, Gaithersburg, MD, USA, and AllCells, Foster City, CA, USA). CD34⁺ cells were selected 1 day after sample collection using the MiniMACS positive selection system (Miltenyi Biotec, Auburn, CA, USA). The purity of CD34⁺ cells in the selected cell population was $97 \pm 0.7\%$ (n = 6). TPO (Genentech, South San Francisco, CA, USA, 50 ng/mL), IL-3 (R&D Systems, Minneapolis, MN, USA, 5 ng/mL), and Flt-3 ligand (R&D Systems, 50 ng/mL) were added to serum-free X-VIVO 20 (BioWhittaker, Walkersville, MD, USA) medium. Immediately after selection, CD34⁺ cells were seeded at 30,000 cells/mL in 25 cm² T-flasks (10 mL of medium per flask) and incubated for 21 days at 37°C in a fully humidified environment containing 5% CO₂ and 5% or 20% O₂, with the balance N₂. The pO₂ levels in both the gas and liquid phases were verified using a blood-gas analyzer (Model 1306, Instrumentation Laboratories, Lexington, MA, USA). Cell densities were maintained between 1 and 2×10^5 cells/mL by dilution with fresh cytokine-containing medium equilibrated at the respective pO₂. To ensure saturation, TPO (50 ng/mL) was added to cultures every 5 days.

Choice of time points for transcription factor studies

Due to the limited number of Mk, especially at the earlier part of a culture, flow cytometry and/or RT-PCR data for transcription factors could be collected on only two days per sample. Based on our earlier results [9] and the assumption that Mk maturation-specific transcription factor upregulation will occur prior to the associated phenotypic changes, days 6 and 9 were chosen for preliminary studies. Although differential transcription factor expression was observed on both days, the differences were not statistically significant at day 6. Therefore, days 9 and 11 were chosen for a second set of studies. In this report, the results from all of the donor samples for a given day (day 6, 9, or 11) have been used for statistical analysis.

One way to obviate the limitation on Mk numbers is to use a megakaryocytic cell line instead of primary cells. However, several recent studies indicate that for molecular studies it is imperative to use primary Mk because significant disparities exist in the results obtained using primary Mk vs Mk cell lines [31,32].

Monoclonal antibodies

Anti-CD41a (P2) and anti-CD42a (SZ1) antibodies were purchased from Coulter/Immunotech (Westbrook, MA, USA). Anti-c-Mpl antibody (BAH-1) [33] was a kind gift from Dr. H. Avraham (Harvard Medical School, Boston, MA, USA). Anti-IL-3R α (CD123) (9F5), anti-Flt3 (CD135)

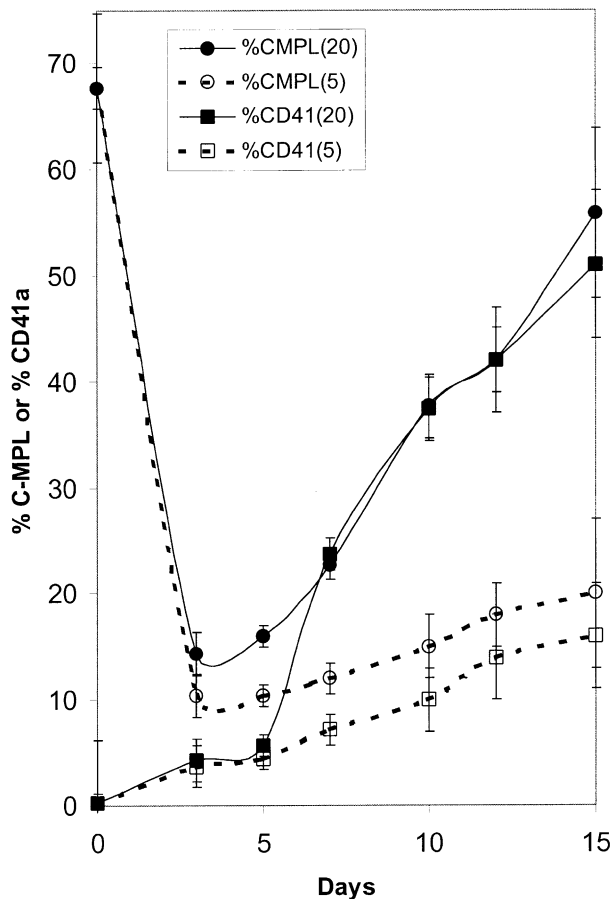


Figure 3. The dynamics of c-Mpl and CD41a expression at 20% and 5% O₂ (n = 10). Culture conditions are described in the legend to Figure 1. Beginning at days 5 and 7, respectively, the percentages of c-Mpl⁺ cells and CD41a⁺ cells were significantly greater at 20% O₂.

(4G8), and anti-NMDAR1 (54.1) antibodies were purchased from BD PharMingen (San Diego, CA, USA). Rat anti-human GATA-1 (N1), rabbit anti-human NF-E2 (C-19), mouse anti-human E2F-1 (KH95), and rat, rabbit, and mouse IgG control antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Flow cytometric detection of CD41a, CD42a, IL-3R, and Flt3

1 to 2 × 10⁵ cells were simultaneously labeled with anti-CD41a-FITC and either anti-CD123-PE (IL-3Rα), anti-CD135-PE (Flt3), or anti-CD42a-PE. A mixture of IgG-FITC and IgG-PE was used as control. Flow cytometry was performed using a FACScan flow cytometer (Becton-Dickinson, San Jose, CA, USA). The fractions of the various cell types were quantitated using quadrant statistics in CELLQuest software (Becton-Dickinson). In order to quantitate the intensities for a particular antigen, histogram statistics from the CELLQuest software were used. Due to the nonGaussian dis-

tribution of the population, the median intensity was chosen to describe a population, rather than the mean intensity. IL-3R content generally exhibited a bimodal distribution, and markers were drawn based on the valley between the two peaks to distinguish the IL-3R⁺ vs IL-3R⁺⁺ cells.

Flow cytometric detection of c-Mpl

1 to 2 × 10⁵ cells were first incubated with goat IgG blocking antibody for 1 hour. Cells were then incubated with non-conjugated mouse anti-human c-Mpl antibody for 30 minutes. Finally, cells were incubated with a goat anti-mouse IgG-FITC antibody for 30 minutes. Initially, we attempted to sequentially stain these cells with anti-c-Mpl antibody and mouse anti-human CD41a antibody. Accomplishing this required a second blocking step with mouse IgG between the two staining protocols. This, however, led to a significant decrease in the ability to detect CD41a, possibly due to steric hindrance. Thus, all subsequent studies (including those presented here) were done with c-Mpl staining alone.

Flow cytometric detection of transcription factors and NMDAR1

Immunofluorescent staining and flow cytometric analysis is a powerful tool for multiparametric analysis of a heterogeneous cell population at the level of individual cells. An additional benefit of flow cytometry is the relatively small number of cells (~100,000) that is required per sample. In order to detect GATA-1, NF-E2, and E2F-1 transcription factors via flow cytometry, a fixation and permeabilization protocol similar to one used for detection of nuclear NF-κB [34] was used. Although NMDAR1 is a cell surface receptor, the particular antibody used to detect this receptor recognizes an intracellular loop between two putative transmembrane regions. Therefore, permeabilization of the cells was also required to detect this antigen. Cells were first fixed and permeabilized by incubation for 15 minutes in 4% paraformaldehyde in phosphate-buffered saline (PBS), pH 7.2, with 0.1% saponin and 0.01 mmol/L HEPES. Cells were washed and incubated with goat IgG blocking antibody for 1 hour in 0.1% saponin with 0.5% bovine serum albumin (BSA). Cells were then incubated with the primary antibody for 30 minutes, followed by incubation with the secondary antibody for 30 minutes. Cells were washed and incubated with mouse IgG for 30 minutes in PBS with 0.5% BSA. Finally, cells were incubated with anti-CD41a-FITC for 30 minutes, also in PBS with 0.5% BSA. The fluorescence intensities for the transcription factors and the NMDAR1 receptor were amplified due to the indirect staining approach.

Reverse-transcriptase polymerase chain reaction (RT-PCR) analysis of Mk-specific transcription factor mRNA levels

Positive selection of CD41a⁺ Mks from days 9 and 11 of culture was carried out using the CELLection Pan Mouse

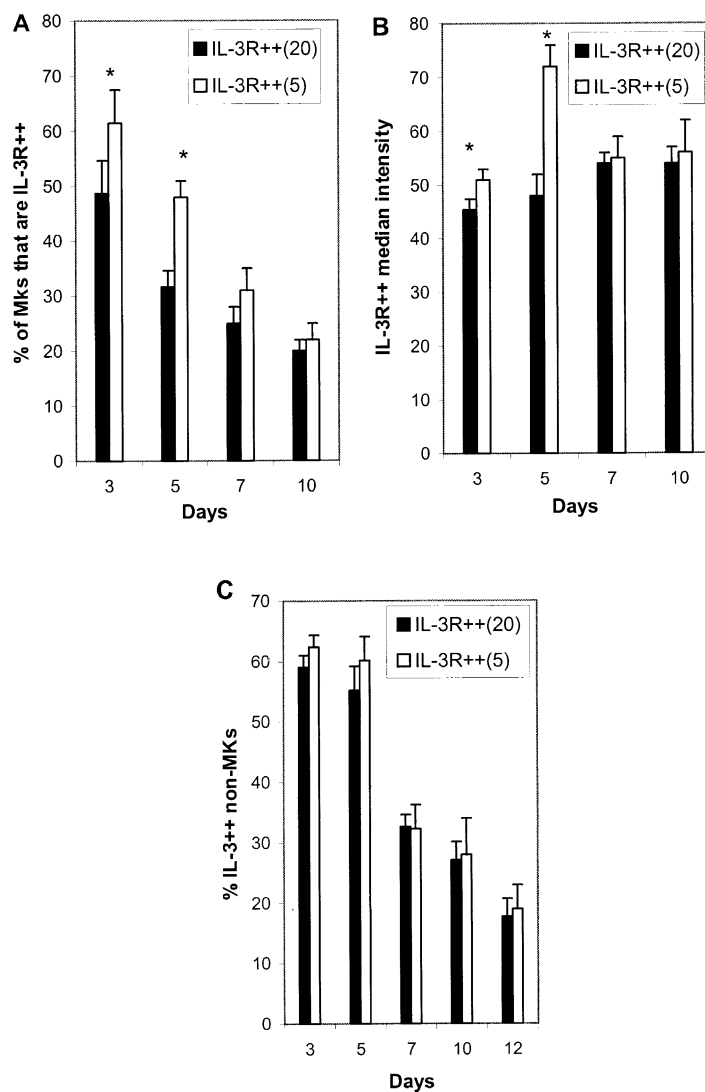


Figure 4. (A) The percentage of Mks that exhibit IL-3R⁺⁺ is higher at 5% O₂ (n = 6). (B) The level of IL-3R on IL-3R⁺⁺ Mks is higher at 5% O₂ (n = 6). (C) The percentage of IL-3R⁺⁺ non-Mks is indifferent between 20% and 5% O₂ cultures (n = 6). Culture conditions are described in the legend to Figure 1. In (B) the median fluorescence intensity of cells incubated with nonspecific antibody was 3 units. * indicates a significant difference ($p < 0.05$) between 20% and 5% O₂ cultures.

IgG kit (DynaL, Lake Success, NY, USA) following the manufacturer's protocol. Total RNA was extracted from an equal number of selected Mks using Tri-Reagent (Sigma, St. Louis, MO, USA) according to the manufacturer's protocol. RNA was reverse transcribed into cDNA with Moloney murine leukemia virus reverse transcriptase (Promega, Madison, WI, USA). The primer pairs used for GATA-1 and NF-E2 have been reported previously [35,36]. Primers for β -actin [37] were included in each set of PCR reactions to normalize the cDNA levels in the PCR products. Thirty cycles of amplification were performed using a program specific for each gene primer set. Reaction products were separated on 2% agarose gels stained with ethidium bromide. No reaction products were detected using RNA samples from which reverse transcriptase had been omitted

(data not shown). Quantification of fold differences in mRNA levels was performed using the Kodak Digital Science Electrophoresis Documentation and Analysis System 120 (Eastman Kodak Co., Rochester, NY, USA) using ϕ X174 DNA—*Hae* III digest as a control.

Statistical analysis

Data are reported as mean \pm standard error of the mean (SEM) for "n" separate donors. Statistical comparisons between 20% and 5% O₂ cultures were obtained using a two-tailed paired Student's *t*-test. The Kruskal-Wallis test [38] was used to determine the statistical significance of day 6 vs day 9 vs day 11 intensities of GATA-1 and NF-E2 expression for a given pO₂. The Kruskal-Wallis test is the non-

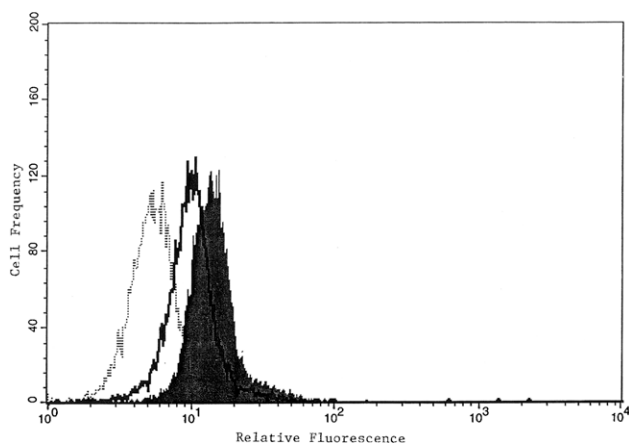


Figure 5. Representative histograms showing higher Flt3 intensity for Mks at 20% O₂ on day 7. Filled histogram = Mks at 20% O₂ (median fluorescence intensity = 15); solid line = Mks at 5% O₂ (median fluorescence intensity = 10); and broken line = control (median fluorescence intensity = 4.5). Culture conditions are described in the legend to Figure 1.

parametric analog of one-way ANOVA and does not assume a normal distribution or constant variance.

Results

CD41a⁺ and CD42a⁺ cells

are present at a higher proportion at 20% O₂

The kinetics of CD41a and CD42a expression in the Mk cultures indicate that both of these Mk-specific markers are present on a higher proportion of cells at 20% O₂ (Fig. 1). All of the CD42a⁺ cells were also CD41a⁺ (data not shown). The differences between the CD41a⁺ and CD42a⁺ fractions are consistent with the observation that CD42a is a later Mk marker [10–12]. The fraction of CD41a⁺ cells that were also CD42a⁺ was not significantly different between 20% and 5% O₂ cultures.

CD41a and CD42a markers

are expressed at a higher level at 20% O₂

Since the levels of CD41a and CD42a per cell increase as Mks mature [11], higher intensities for these proteins would suggest more extensive maturation. CD41a (Fig. 2A) and CD42a (Fig. 2B) are expressed at a higher density at 20% O₂ in Mks up to day 12 of culture. After day 15, the levels of CD41a and CD42a did not differ between 20% and 5% O₂. It has been shown that apoptosis leads to shedding of several surface proteins [39]. Since a significant fraction of the Mks undergo apoptosis between days 15 and 21 of culture [9], it is possible that shedding of CD41a and CD42a takes place during this time.

c-Mpl expression is significantly affected by pO₂

On day 0, 67% of the CD34⁺ cells were c-Mpl⁺ (Fig. 3). However, by day 3 a much lower fraction of cells expressed

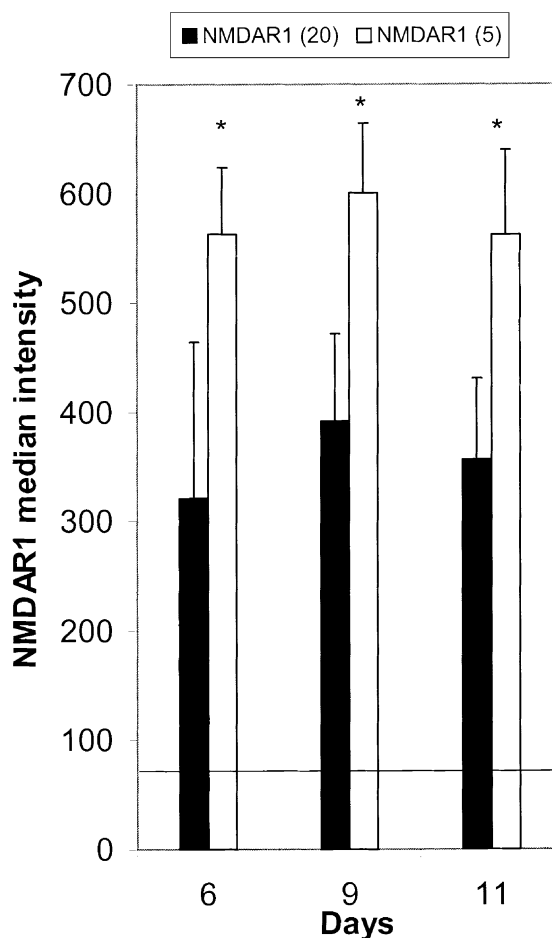


Figure 6. The level of NMDAR in Mks is higher at 5% O₂ (n = 6 for day 6; n = 9 for day 9; n = 3 for day 11). Culture conditions are described in the legend to Figure 1. The horizontal line indicates the median fluorescence intensity of cells incubated with nonspecific antibody. * indicates a significant difference ($p < 0.05$) between 20% and 5% O₂ cultures.

c-Mpl. From day 5 onward the percentage of c-Mpl⁺ cells was significantly higher at 20% O₂. At day 15, the percentage of c-Mpl⁺ cells at 20% O₂ was 2.8 times higher than that at 5% O₂. After day 5 in 20% O₂ culture and after day 7 in 5% O₂ culture, c-Mpl expression showed similar kinetics as CD41a expression. Between days 3 and 15, c-Mpl intensities at 20% O₂ were either statistically higher (~1.1-fold of that at 5% O₂ on days 3 and 10, $p < 0.05$) or similar to the c-Mpl intensities at 5% O₂ (data not shown).

The proportion and IL-3R

intensity of IL-3R⁺⁺ Mks are higher at 5% O₂

At days 3 and 5, the percentage of IL-3R bright (IL-3⁺⁺) CD41a⁺ cells was significantly higher under 5% O₂ (Fig. 4A). At these days, and especially on day 5, the IL-3R level on IL-3R⁺⁺ cells within the Mk population was also higher at 5% O₂ (Fig. 4B). Between days 7 and 10, the proportion and IL-3R levels of IL-3R⁺⁺ Mks in the 20% and 5% cultures were similar. Beyond day 10, IL-3R⁺⁺ Mks were not

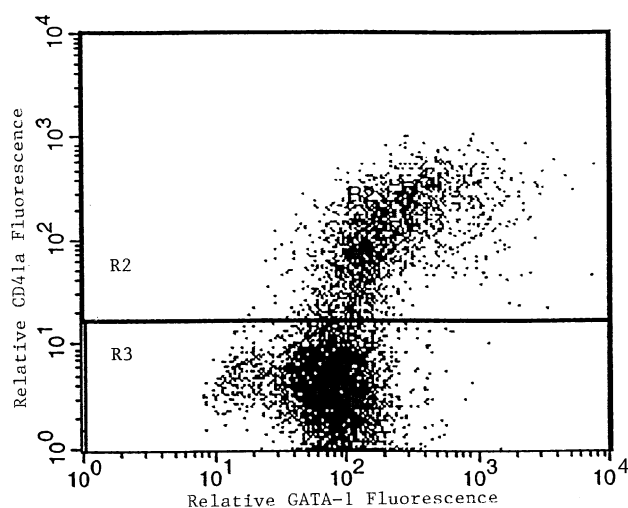


Figure 7. Representative dot plot showing GATA-1 vs CD41a fluorescence intensities at day 9 for a culture conducted at 5% O₂. Other culture conditions are as described in the legend to Figure 1. The regions used to discriminate the CD41a⁺ (R2) and CD41a⁻ (R3) populations are indicated.

observed. In the CD41a⁻ population, the % IL-3R⁺⁺ cells also decreased with time, but was similar in 20% and 5% O₂ cultures (Fig. 4C). The intensity of IL-3R on CD41a⁻ IL-3R⁺⁺ cells was also similar between 20% and 5% O₂ cultures (data not shown).

Flt3 expression is affected by pO₂ in Mk and non-Mk cells

Normal bone marrow mononuclear cells have an average of 40 Flt3 molecules per cell, which is 100-fold less than the c-kit density [40]. A representative histogram for Flt3 intensity in Mks at 20% and 5% O₂ is shown in Figure 5. At days 3, 5, and 7 the median intensities of Flt3 in Mks at 20% O₂ were 14 ± 1, 10 ± 0.8, and 14 ± 3, respectively, and at 5% O₂ were 13 ± 0.3, 9 ± 1, and 9 ± 3, respectively. For non-Mk cells, at days 3, 5, and 7 the median intensities of Flt3 at 20% O₂ were 12 ± 1, 10 ± 0.7, and 12 ± 2, respectively, and at 5% O₂ were 12 ± 0.5, 9 ± 0.8, and 9 ± 1, respectively. For days 3, 5, and 7 the median intensity for the isotype control was 5 ± 1. On day 7, the Flt3 intensity at 20% O₂ was higher than at 5% O₂ for both CD41a⁺ and CD41a⁻ cells ($p < 0.05$). Beyond day 7 of culture, the Flt3 level was indistinguishable from the control.

Glutamate receptor is

expressed at a higher level in Mks under 5% O₂

Figure 6 shows the median intensities of NMDAR1 protein levels within the CD41a⁺ Mk population. The average median density of this receptor on Mks at 5% vs 20% O₂ was 1.8-, 1.5-, and 1.5-fold higher at days 6, 9, and 11, respectively.

GATA-1 is expressed at a higher level in Mks at 20% O₂

A representative dot plot illustrating GATA-1 and CD41a double staining, as well as the regions used to discriminate

between CD41a⁺ and CD41a⁻ cells, is shown in Figure 7. A representative histogram for GATA-1 intensity in Mks at 20% and 5% O₂ is shown in Figure 8A. Figure 9A shows the median intensity of GATA-1 within the CD41a⁺ Mk population under 20% and 5% O₂. The average median GATA-1 levels at 20% O₂ at days 6, 9, and 11 were 1.2-, 1.6-, and 2.2-fold higher, respectively, than at 5% O₂. At days 9 and 11, the difference between GATA-1 levels under 20% and 5% O₂ was statistically significant ($p < 0.05$). Based on the Kruskal-Wallis test, the GATA-1 level decreased between days 6 and 11 at 5% O₂ ($p < 0.05$).

NF-E2 is expressed at a higher level in Mks at 20% O₂

A representative histogram for NF-E2 intensity in Mks at 20% and 5% O₂ is shown in Figure 8B. Figure 9B shows the median intensity of NF-E2 within the CD41a⁺ Mk population under 20% and 5% O₂. The average median NF-E2 levels at 20% O₂ at days 6, 9, and 11 were 1.1-, 1.6-, and 2.8-fold higher, respectively, than at 5% O₂. At days 9 and 11, the difference between NF-E2 levels under 20% and 5% O₂ was statistically significant ($p < 0.05$). Kruskal-Wallis test results indicate that between day 6 and day 11, NF-E2 levels increased at 20% O₂, whereas at 5% O₂ the NF-E2 levels decreased over the same time frame ($p < 0.05$).

Greater mRNA level of

GATA-1 and NF-E2 in Mks at 20% O₂

RT-PCR data indicate that the mRNA levels of GATA-1 and NF-E2 were substantially higher at 20% O₂ compared to 5% O₂ on days 9 and 11. At days 9 and 11 the GATA-1 mRNA level (relative to β-actin) was 1.9- and 3.4-fold higher, respectively, at 20% O₂ in one representative experiment (Fig. 10A). The NF-E2 mRNA level (relative to β-actin) was 1.3- and 1.6-fold higher at 20% O₂ on days 9 and 11, respectively, in the same experiment (Fig. 10B). Similar trends were observed for both transcription factors in replicate experiments ($n = 4$ for day 9, $n = 3$ for day 11).

E2F-1 is expressed at a higher level in Mks under 20% O₂

A representative histogram for E2F-1 intensity in Mks at 20% and 5% O₂ is shown in Figure 8C. On day 6, the E2F-1 median fluorescence intensities of Mks at 20% and 5% O₂ were 509 ± 50 and 337 ± 55, respectively, vs 20 ± 5 for control IgG. The difference between the median intensities at 20% vs 5% O₂ is significant ($p < 0.05$). On day 9, the intensities under 20% and 5% O₂ were 393 ± 55 and 355 ± 52, respectively. Therefore, the elevated level of E2F-1 at 20% O₂ did not persist until day 9.

Discussion

As a first step to elucidate the mechanisms regulating the modulation of megakaryocytopoiesis by pO₂, we have undertaken a comprehensive kinetic analysis of Mk-related

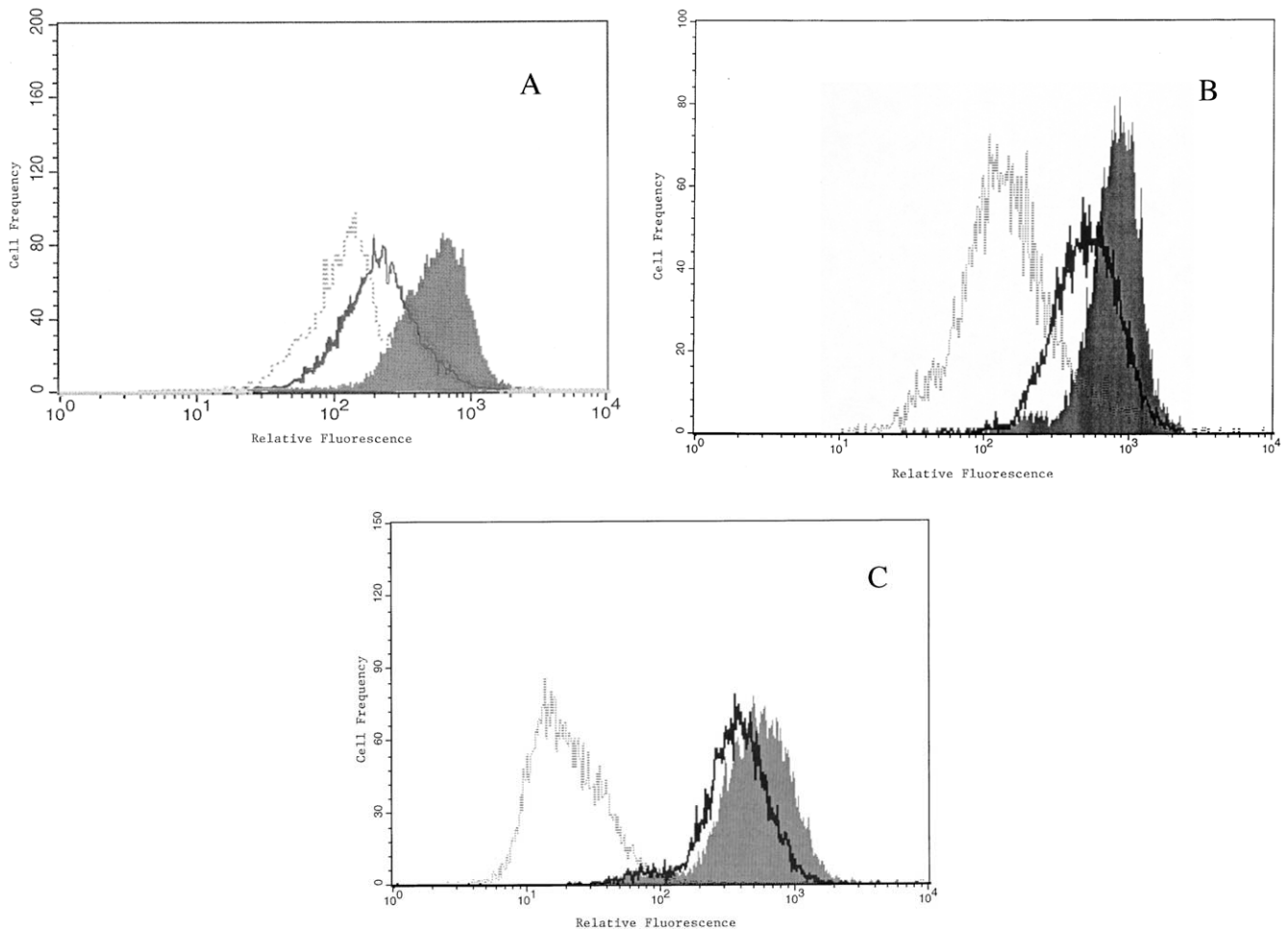


Figure 8. Representative histograms showing higher intensity of (A) GATA-1 (Day 9), (B) NF-E2 (Day 9), and (C) E2F-1 (Day 6) for Mks at 20% O₂. Filled histogram = Mks at 20% O₂; solid line = Mks at 5% O₂; and broken line = control. The respective median fluorescence intensities for the 20% O₂, 5% O₂, and control histograms shown are (A) 537, 231, and 151; (B) 910, 540, and 150; and (C) 550, 420, and 20. Culture conditions are described in the legend to Figure 1.

surface receptor and transcription factor expression at 20% and 5% O₂. pO₂ significantly affects the expression of CD41a, CD42a, and c-Mpl throughout the first 15 days of culture. The percentages of CD41a⁺ cells and CD41a⁺CD42a⁺ cells were significantly higher at 20% O₂ (Fig. 1). The cell surface density of these two proteins was also higher at 20% O₂ (Fig. 2), indicating a more advanced stage of Mk maturation at 20% O₂ [11].

TPO is the primary growth factor regulating Mk differentiation as well as proliferation [41]. *c-mpl* transcripts are present in Mks, platelets, and CD34⁺ cells [42]. We found that beyond day 5 at 20% O₂ and beyond day 7 at 5% O₂, the percentage of c-Mpl⁺ cells was essentially equal to the percentage of CD41a⁺ cells, indicating that all c-Mpl⁺ cells are CD41a⁺ and vice versa (Fig. 3).

In contrast to TPO, which augments all stages of megakaryocytopoiesis, IL-3 is a potentiator of Mk progenitors but not of mature Mks [43]. Testa et al. [44] have shown that within the Mk lineage, IL-3R expression declines pro-

gressively with maturation. In contrast to c-Mpl, IL-3R on Mks appears to be affected by pO₂ only during the early part of a culture (Fig. 4). The lack of any effect of pO₂ on IL-3R⁺⁺ levels for non-Mks suggests a Mk lineage-specific effect of pO₂ on this receptor. It has been suggested that IL-3 has an inhibitory effect on Mk maturation events such as polyploidization [45]. The higher percentage and fluorescence intensity of IL-3R⁺⁺ Mks at 5% O₂ may, therefore, be related to the less mature phenotype of Mks at 5% O₂ [9]. In contrast to the situation for IL-3R, the higher intensity of Flt3 under 20% at day 7 in Mks and non-Mks alike suggests an across-lineage effect of pO₂ on this receptor.

Sattler et al. [46] have shown that stimulation by TPO and IL-3 induces a rapid and sustained increase in the level of reactive oxygen species. When growth factor-deprived MO7e (a megakaryocytic cell line) cells were exposed to hydrogen peroxide, molecular events usually associated with growth factor signaling such as tyrosine phosphorylation of STAT5 and SHC, c-Fos gene expression, and G1- to

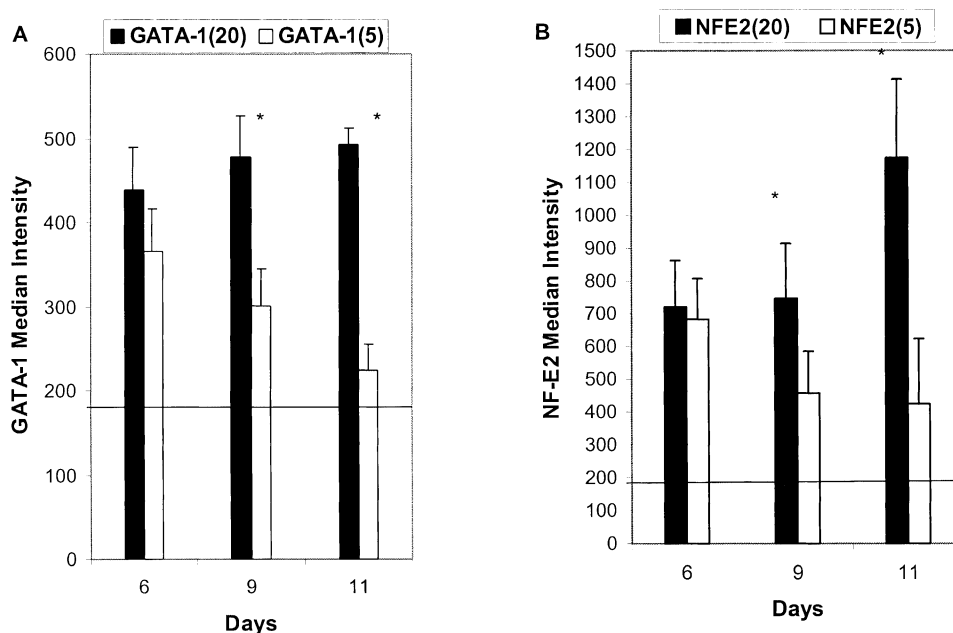


Figure 9. The levels of (A) GATA-1 and (B) NF-E2 in Mks are higher at 20% O₂ (n = 6 on day 6; n = 9 on day 9; n = 3 on day 11). Culture conditions are described in the legend to Figure 1. The horizontal line indicates the fluorescence intensity of cells incubated with nonspecific antibody. * indicates a significant difference ($p < 0.05$) between 20% and 5% O₂ cultures.

S-phase transition were observed [46]. Our results indicate that, compared to the magnitude of phenotypic differences observed at 20% vs 5% O₂, the change in growth factor receptor levels is modest. Perhaps direct modulation of downstream signaling events is in part responsible for the profound effects of pO₂ on the Mk phenotype.

Genever et al. [15] have recently shown that NMDAR1 is expressed by primary human Mks and the megakaryoblastic cell line MEG-01. In the presence of a NMDAR antagonist, the PMA-induced increase in CD41a expression and adhesion of MEG-01 cells was suppressed. However, immunolocalization studies with rat bone marrow indicate that acetylcholine esterase-positive Mks express NMDAR1 regardless of maturational stage [15]. Nothing is known about the expression kinetics of NMDAR1 during megakaryocytopoiesis or the role that growth factors and other environmental factors play on the expression of this receptor in Mks. We show that NMDAR1 is expressed at a higher level in Mks at 5% O₂ and that both the absolute and relative levels between 20% and 5% O₂ do not change between days 6 and 11 (Fig. 6), even though there is substantial Mk maturation during this time period, especially at 20% O₂ [9].

It has been postulated that, during cell differentiation, cytokines provide a permissive (proliferation and/or survival) function, whereas nuclear transcription factors play a deterministic role [16]. Both GATA-1 and NF-E2 protein and mRNA levels were higher under 20% vs 5% O₂ (Figs. 8–10). This suggests that 20% O₂ increases NF-E2 and GATA-1

transcript production and/or mRNA stability, which leads to higher levels of NF-E2 and GATA-1 protein production. GATA-1 knockout mice show a reduction in NF-E2 mRNA level, suggesting that GATA-1 may play a regulatory role in NF-E2 transcription [26]. Thus, pO₂ modulation of GATA-1 and NF-E2 may be related events. Lower GATA-1 and NF-E2 expression at 5% O₂ was not due to a dilution of the mature Mks by an increased number of immature Mks, since the number of immature Mks was also higher at 20% O₂ ([9] and data not shown). Also, during the time period of the transcription factor studies (days 6–11), GATA-1 and NF-E2 levels at 5% O₂ actually decreased, whereas at 20% O₂ these levels either remained unchanged (GATA-1) or substantially increased (NF-E2), even though Mk maturation increased with time at both pO₂ levels ([9] and data not shown). The differential expression of GATA-1 and NF-E2 at different pO₂, therefore, appears to be due to modulation by pO₂ rather than as a consequence of having a larger proportion of mature Mks at 20% O₂.

In contrast to GATA-1 and NF-E2, greater expression of E2F-1 at 20% O₂ was observed only at the earliest culture time assayed. Since Mk numbers are greater at 20% O₂, a higher level of E2F-1 under 20% O₂ at day 6 of culture may suggest a role for E2F-1 in pO₂-mediated Mk proliferation or early differentiation.

Antioxidant response element (ARE)-mediated expression of genes encoding detoxifying enzymes is an important cellular protection mechanism against oxidative stress [47]. AREs have high similarity to the NF-E2 sequence motif [48].

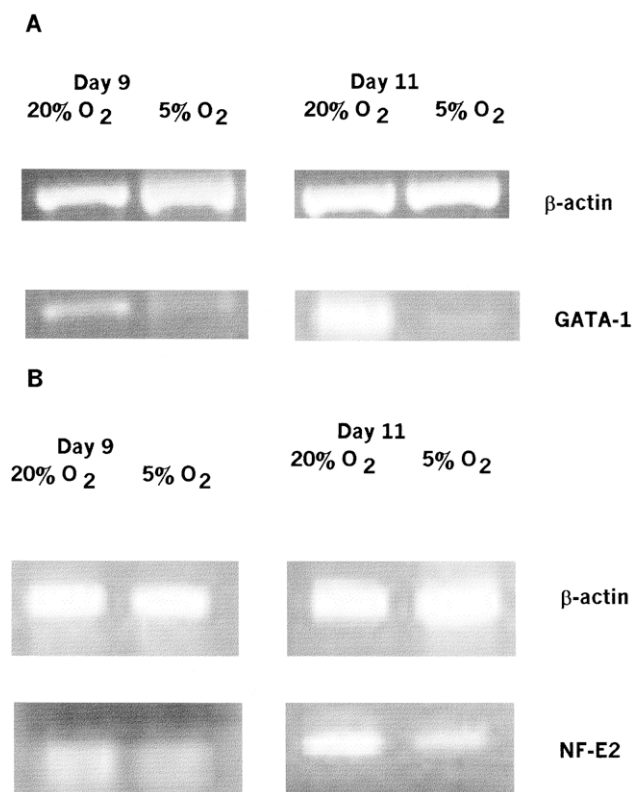


Figure 10. Representative RT-PCR data showing that mRNA levels (relative to those of β -actin) of (A) GATA-1 and (B) NF-E2 are higher at 20% O_2 . Culture conditions are described in the legend to Figure 1.

Recently, several proteins structurally similar to p45 and capable of binding to the NF-E2 consensus binding sequence have been identified, including Nrf2, which controls the expression of electrophile and oxidative stress-inducible proteins [49]. Oxidative stress agents appear to induce gene expression by posttranscriptionally increasing Nrf2 protein levels, and by enhancing the DNA binding activity of Nrf2 in its heterodimeric form to AREs and closely related stress-responsive elements [49]. MafG is the predominant small Maf protein involved in thrombopoiesis *in vivo*, and in the absence of MafG proplatelet formation is suppressed and thrombocytopenia ensues [50]. This result is of interest because hydrogen peroxide induces expression of a MafG homolog [51]. It is therefore conceivable that the higher oxidative stress at 20% O_2 leads to a higher level of NF-E2 (p45/MafG heterodimer) production in Mks that upregulates expression of genes involved in Mk maturation, as well as other oxidative stress-inducible and detoxifying genes. As to the relationship between GATA-1 and pO_2 , the addition of hydrogen peroxide increases the expression of GATA proteins in EPO-producing cells in a dose-dependent manner [52]. Interestingly, expression of the hypoxia-inducible erythropoietin (EPO) gene is significantly inhibited by GATA-1, -2, or -3 [53]. These reports put forward the hypothesis that GATA proteins regulate the EPO gene through an oxygen sensor.

Perhaps a similar regulatory mechanism is also at work in the case of GATA-1 activation of Mk-specific genes.

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