Cell Death and Differentiation (2010) 17, 1842–1854

www.nature.com/cdd

p22^{phox}-dependent NADPH oxidase activity is required for megakaryocytic differentiation

JL Sardina[1], G López-Ruano[1], LI Sánchez-Abarca[2], JA Pérez-Simón[2], A Gaztelumendi[3], C Trigueros[3], M Llanillo[1], J Sánchez-Yagüe[1] and A Hernández-Hernández*,[1]

Transient reactive oxygen species (ROS) production is currently proving to be an important mechanism in the regulation of intracellular signalling, but reports showing the involvement of ROS in important biological processes, such as cell differentiation, are scarce. In this study, we show for the first time that ROS production is required for megakaryocytic differentiation in K562 and HEL cell lines and also in human CD34$^+$ cells. ROS production is transiently activated during megakaryocytic differentiation, and such production is abolished by the addition of different antioxidants (such as *N*-acetyl cysteine, trolox, quercetin) or the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase inhibitor diphenylene iodonium. The inhibition of ROS formation hinders differentiation. RNA interference experiments have shown that a p22^{phox}-dependent NADPH oxidase activity is responsible for ROS production. In addition, the activation of ERK, AKT and JAK2 is required for differentiation, but the activation of phosphatidylinositol 3-kinase and c-Jun N-terminal kinase seems to be less important. When ROS production is prevented, the activation of these signalling pathways is partly inhibited. Taken together, these results show that NADPH oxidase ROS production is essential for complete activation of the main signalling pathways involved in megakaryocytopoiesis to occur. We suggest that this might also be important for *in vivo* megakaryocytopoiesis.
Cell Death and Differentiation (2010) **17,** 1842–1854; doi:10.1038/cdd.2010.67; published online 4 June 2010

Eukaryotic cells have to deal constantly with the formation of reactive oxygen species (ROS) as a consequence of their aerobic metabolism. ROS production may have deleterious effects on cells, and it has been traditionally related to ageing and to a number of degenerative diseases.[1] However, it is now accepted that ROS have an important role in regulating signal transduction pathways[2] and gene expression,[3] although the molecular mechanisms are not fully understood. ROS are generated in nearly all types of cells of multi-cellular organisms, either as a consequence of mitochondrial metabolism or as by-products of different enzymatic reactions. The main non-mitochondrial sources of ROS are nicotinamide adenine dinucleotide phosphate (NADPH) oxidases (Nox).[4] These proteins are membrane-associated multi-protein complexes that produce superoxide (O_2^-). The first complex to be described was the phagocyte NADPH oxidase, which produces huge amounts of superoxide and other ROS that are crucial for host defence. The catalytic subunit of this complex (gp91^{phox} or Nox2) is tightly bound to the p22^{phox} protein, forming cytochrome b_{558}, which in resting cells resides in intracellular vesicles, but upon stimulation, migrates to the plasma membrane and, together with the cytosolic subunits (p47^{phox}, p67^{phox}, p40^{phox} and the GTPase Rac), forms an active NADPH oxidase complex.[5] There are six homologues of Nox2 (namely Nox1, Nox3, Nox4, Nox5, Duox1 and Duox2); each has a specific pattern of expression, but their specific roles remain to be unravelled.[4]

A few recent reports have described the relevance of NADPH oxidase ROS production in the differentiation of different kind of cells.[6,7] Therefore, it is tempting to speculate that NADPH ROS production might be a general mechanism common to all types of cell differentiation. However, to confirm this, further studies should be carried out on other types of cell differentiation.

Haematopoiesis is probably one of the best examples of cell differentiation because haematopoietic stem cells (HSCs) maintain differentiation of all blood lineages throughout the life of the individual. ROS seem to be involved in the regulation of the lifespan[8] and self-renewal potential of HSCs.[9] In addition, it is known that haematopoietic cytokine signalling is accompanied by ROS formation.[10] In addition, ROS can contribute to leukaemic cell transformation.[11] Bearing the foregoing in mind, it would be reasonable to assume that ROS production could have a direct role in haematopoietic differentiation. Nevertheless, there are almost no studies addressing this issue, with just one report describing the differentiation of a promonocytic cell line into macrophages.[12] Moreover, to our knowledge, there are no reports exploring

[1]Department of Biochemistry and Molecular Biology, University of Salamanca, Salamanca, Spain; [2]Department of Hematology, Hospital Clínico Universitario de Salamanca, Salamanca, Spain and [3]Fundación Inbiomed, Foundation for Stem Cell Research, Haematopoietic and Mesenchymal Cell Department, San Sebastián, Spain
*Corresponding author: A Hernández-Hernández, Departamento de Bioquímica y Biología Molecular. Lab. 106, Edificio Departamental, Plaza Doctores de la Reina s/n, Salamanca 37007, Spain. Tel: +34 923 294 465; Fax: +34 923 294 579; E-mail: angelhh@usal.es
Keywords: haematopoiesis; megakaryocytic differentiation; NADPH oxidase; p22phox protein; reactive oxygen species
Abbreviations: 7-AAD, 7-aminoactinomycin D; APC, allophycocyanin; DCFDA, 2′,7′-dichlorofluorescein diacetate; DPI, diphenylene iodonium; FITC, fluorescein isothiocyanate; G-CSF, granulocyte colony-stimulating factor; GpA, glycophorin A; HSC, haematopoietic stem cell; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide; NAC, *N*-acetyl cysteine; NADPH oxidase (Nox), nicotinamide adenine dinucleotide phosphate oxidase; PE, phycoerythrin; PerCP, peridinin chlorophyll protein; PI, propidium iodide; PMA, phorbol 12-myristate 13-acetate; ROS, reactive oxygen species; shRNA, short hairpin RNA; SOD, superoxide dismutase; TMRE, tetramethyl rhodamine ethyl ester; TPO, thrombopoietin; Trolox, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid
Received 08.10.09; revised 28.4.10; accepted 29.4.10; Edited by RA Knight; published online 04.6.10

the possible role of ROS and NADPH oxidases in the differentiation of HSCs or in other haematopoietic lineages other than macrophages, including megakaryocytic differentiation, that is, the process of maturation of megakaryocytes from HSCs that releases circulating platelets.[13] K562 and HEL cells are commonly used to study different aspects related to megakaryocytopoiesis because they undergo megakaryocytic differentiation in response to phorbol esters.[14,15] Accordingly, our goal was to study the putative role of ROS in megakaryocytic differentiation using both cell lines and human $CD34^+$ cells, to gain insight into the principles that might help in our understanding of how haematopoiesis is regulated and how NADPH oxidase ROS production helps general cell differentiation.

Our data show that ROS production is required for this differentiation process in both cell lines and human HSCs. We also show that ROS production is due to a $p22^{phox}$-dependent NADPH oxidase activity, and that ROS are required to achieve complete activation of the signalling pathways that lead to megakaryocytopoiesis.

Results

ROS production is required for megakaryocytic differentiation. ROS formation in response to phorbol esters has been described in different systems,[16] including K562 cells overexpressing NADPH oxidase,[17] but not in HEL cells. Accordingly, we analysed intracellular ROS production by flow cytometry in K562 and HEL cells under phorbol 12-myristate 13-acetate (PMA) treatment. A rapid and significant increase in ROS levels was observed in both cells lines, with a peak within the first hour. In the presence of the antioxidant quercetin, ROS levels were significantly lower (Figure 1a). To test whether ROS were important for megakaryocytic differentiation, cell differentiation experiments were carried out in the presence or absence of quercetin. The antioxidant partially blocked the differentiation of both cells lines: (1) The level of the megakaryocytic markers (CD41 and CD61) was significantly lower in the presence of quercetin, and moreover, (2) the decrease in the expression in the erythrocytic marker glycophorin A (GpA) that accompanies megakaryocytic differentiation was also prevented in both cell lines (Figure 1b). (3) After PMA treatment, the cells showed megakaryocytic morphological features: larger cells with polylobulated or polysegmented nuclei and a basophilic cytoplasm. However, quercetin avoided the acquisition of these morphological features in both cell lines (Figure 1c and d). Quercetin did not affect cell viability (Supplementary Figure 1a) or cell proliferation (Supplementary Figure 1b). Therefore, quercetin was indeed affecting cell differentiation.

Other antioxidants (such as *N*-acetyl-L-cysteine (NAC) and trolox) also reduced the intracellular levels of ROS (Supplementary Figure 1c), and also hampered megakaryocytic differentiation (Supplementary Figure 1d and e) without affecting cell viability (Supplementary Figure 1f) or cell proliferation (Supplementary Figure 1g). The fact that three different antioxidants, with different mechanism of action[18–20] hamper megakaryocytic differentiation suggests that ROS increase is indeed necessary for cell differentiation.

To test how important this initial burst of ROS is for differentiation, we decided to wash out the PMA stimulus shortly after induction. The results show that, even with only 2 h of PMA treatment, there was a significant increase in the megakaryocytic markers (CD41 and CD61) and a significant decrease in the erythrocytic marker GpA in both cell lines. Moreover, the levels of these markers were very similar to those reached when PMA is not eliminated (Supplementary Figure 2). This suggests that once the ROS burst has occurred, cells seem to be committed to differentiation.

To determine whether this observation was biologically meaningful, we carried out differentiation experiments with human $CD34^+$ cells. Recombinant human thrombopoietin (TPO) induced a rapid increase in intracellular ROS. In the presence of 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), ROS levels were significantly lower (Figure 2b). Trolox and NAC inhibited the acquisition of the megakaryocytic morphological features (Figure 2c). Furthermore, the megakaryocytic marker levels (CD41, CD42b and CD61) were significantly lower in the presence of antioxidants (Figure 2d and e). Cell viability was even higher when cells were differentiated in the presence of antioxidants (Supplementary Figure 3), which means that the antioxidants have indeed an effect on cell differentiation.

Taken together, these data support the notion that ROS production is required for megakaryocytic differentiation of both cell lines and human $CD34^+$ cells, and strongly suggest that ROS production must also be important for *in vivo* megakaryocytopoiesis.

If ROS production was an essential event in the triggering of megakaryocytic differentiation, it would be expected that treatment with oxidising agents such as H_2O_2 might induce, at least to some extent, the expression of megakaryocytic markers. This hypothesis proved to be true, because H_2O_2 induced the expression of some of megakaryocytic markers we analysed in both cell lines (data not shown).

Involvement of NADPH oxidase in ROS production and megakaryocytopoiesis. We next studied the origin of ROS. A likely possibility is that ROS production would be a consequence of some NADPH oxidase activated during differentiation. To test this hypothesis, we first measured NADPH oxidase activity. PMA induced an increase in NADPH oxidase activity in a dose-dependent manner (Supplementary Figure 4a). This increase was inhibited by one of the most common NADPH oxidase activity inhibitors, namely diphenylene iodonium (DPI) (Figure 3a). Moreover, in the presence of DPI, intracellular ROS levels were significantly lower in K562 and HEL cells (Figure 3b). In addition, supplementary analyses showed that DPI treatment was also hindering differentiation: (1) the compound prevented the increase in cell size (Figure 3c) and acquisition of the megakaryocytic morphological features (Figure 3d); (2) the increase in the megakaryocytic markers (CD41 and CD61) and the decrease in the erythrocytic marker GpA were partially inhibited by DPI (Figure 3e and Supplementary Figure 4b); (3) DPI completely inhibited the formation of polyploid cells in both cell lines (Figure 3f). The shape of the peaks (Figure 3f) suggests that DPI would induce the accumulation of cells in the G_2-M phase, as has

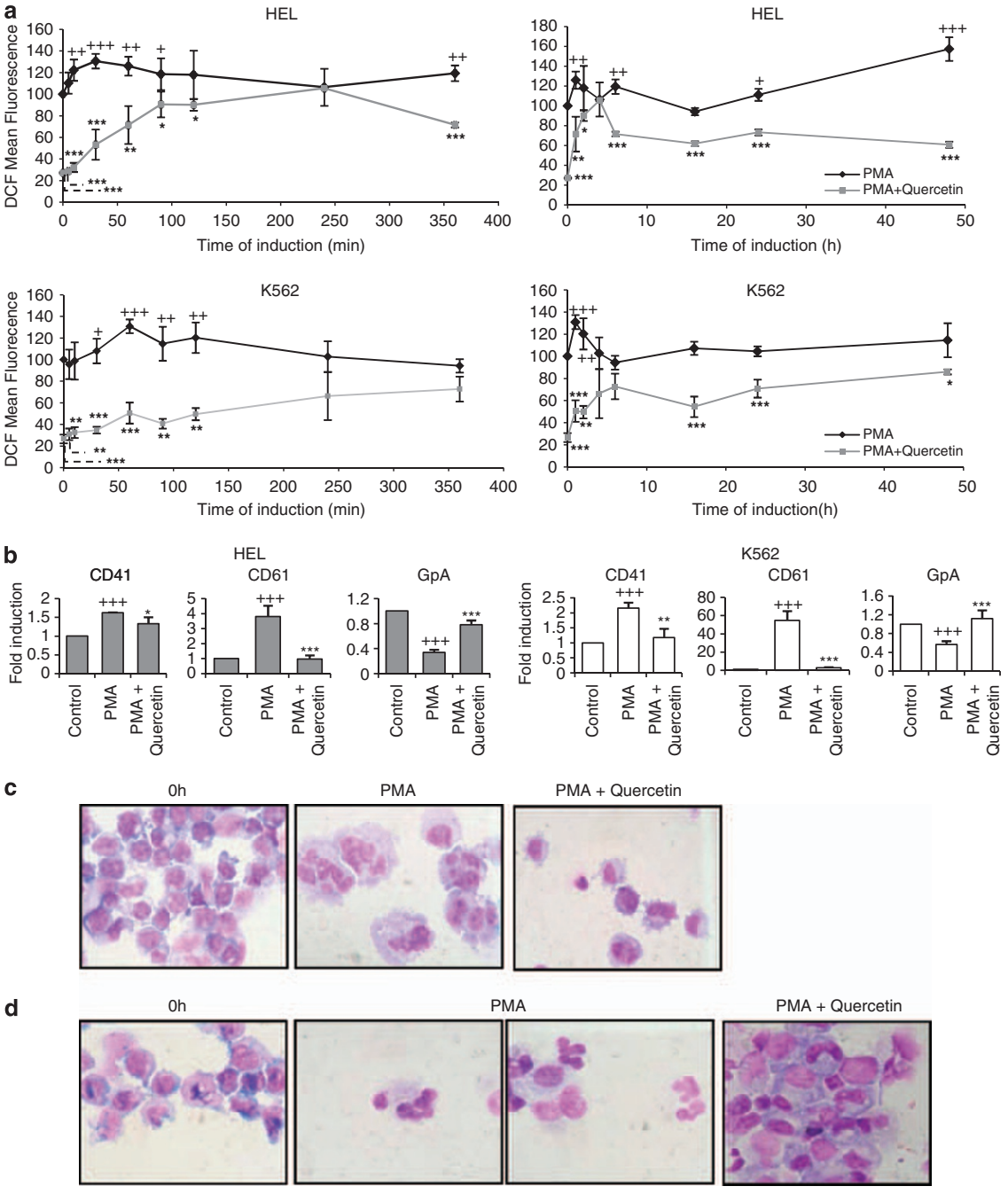


Figure 1 ROS production is required for megakaryocytic differentiation of K562 and HEL cells. (**a**) HEL and K562 cells were stimulated with 20 nM PMA, and intracellular ROS production was measured by flow cytometry in cells labelled with DCFDA in the presence or absence of 100 μM quercetin at different time points. ROS levels were normalised to those of untreated cells. (**b**) Quercetin hindered megakaryocytic differentiation; the results show the megakaryocytic markers (CD41 and CD61) or erythrocytic marker (GpA) fold increase with respect to control cells in HEL and K562 cells after 48 h of differentiation. For PMA-differentiated cells, control cells were treated with vehicle (dimethyl sulphoxide or DMSO) diluted 10^5 times. For PMA + quercetin treatment, control cells were treated with DMSO + 100 μM quercetin. The results are the means ± S.D. of four different experiments. $^{+++}P<0.001$, $^{++}P<0.01$ and $^{+}P<0.05$ reflect significant differences of PMA-treated cells with respect to undifferentiated cells. $^{***}P<0.001$, $^{**}P<0.01$ and $^{*}P<0.05$ reflect significant differences of PMA + quercetin treatment when compared with PMA-treated cells. Cell staining with May–Grünwald–Giemsa of HEL (**c**) and K562 cells (**d**) treated with PMA for 96 h in the presence or absence of 100 μM quercetin

been shown recently.[21] This would imply that ROS production might also be involved in cell-cycle progression, thus being crucial for endomitosis. In this sense, while this paper was in preparation, a report appeared describing the potential role of NADPH oxidases in megakaryocyte polyploidisation,[22] in agreement with our results (Figure 3f). Under our experimental condition, DPI did not affect cell viability (Supplementary Figure 4c) or mitochondrial potential (Supplementary Figure 4d). Moreover, a different NADPH oxidase inhibitor, apocynin, also hampered differentiation,

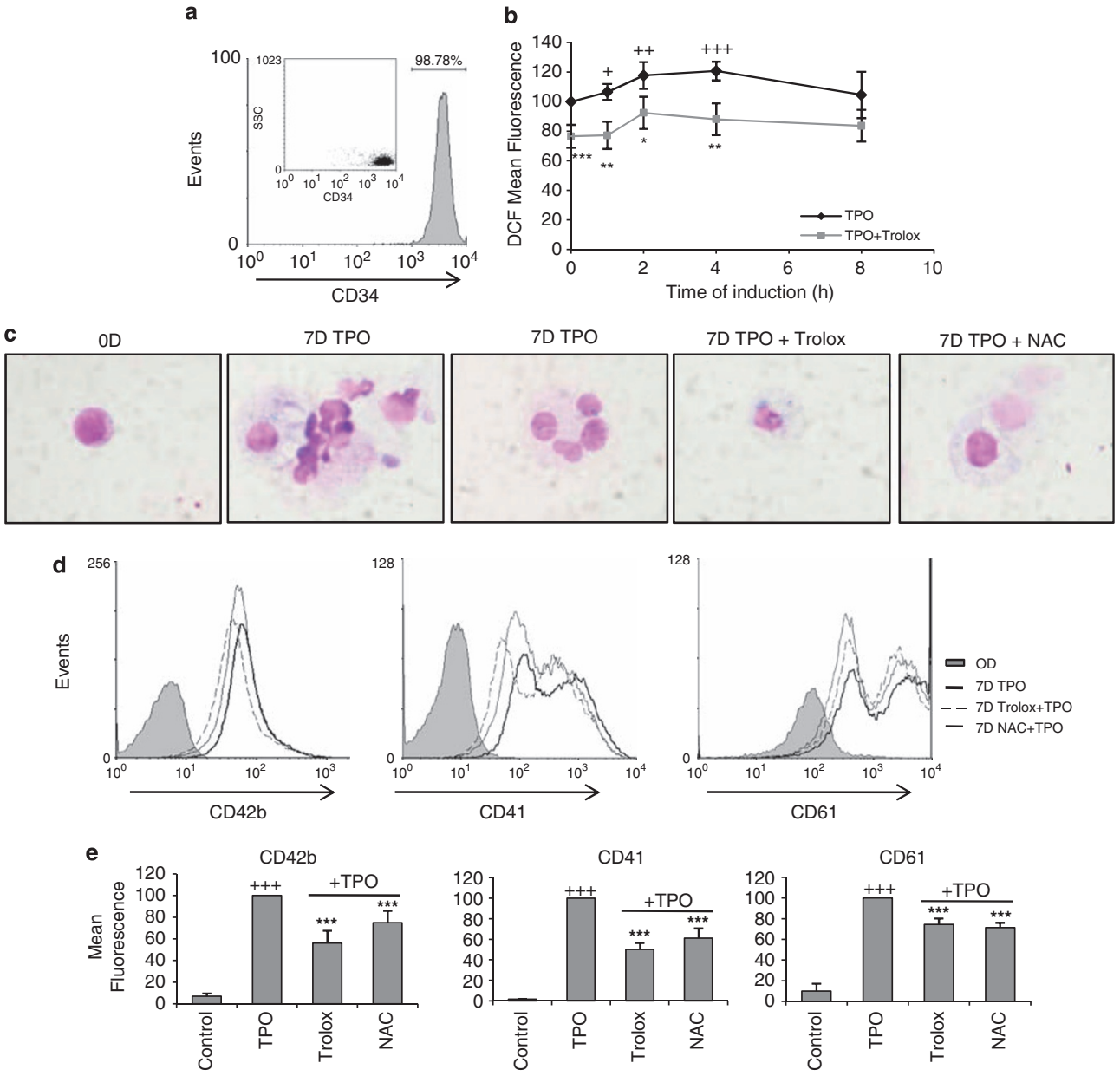


Figure 2 ROS production is required for megakaryocytic differentiation of human CD34$^+$ cells. Human CD34$^+$ cells were isolated from peripheral blood. (**a**) CD34$^+$ cell purity was $>98\%$. (**b**) Intracellular ROS production in response to 100 ng/ml TPO was measured by flow cytometry in cells labelled with DCFDA in the presence or absence of 10 μM Trolox. ROS levels were normalised to those of untreated cells. Cells were differentiated by treatment with 100 ng/ml TPO in the presence or absence of 10 μM Trolox or 100 μM NAC for 7 days and the following analyses were carried on: (**c**) cell staining with May–Grünwald–Giemsa; (**d**) megakaryocytic differentiation marker levels (CD42b, CD41 and CD61), a representative experiment is shown; (**e**) the marker levels (means ± S.D. of four different experiments) are shown. $^{+++}P<0.001$, $^{++}P<0.01$ and $^{+}P<0.05$ reflect significant differences when compared with undifferentiated control cells; $^{***}P<0.001$, $^{**}P<0.01$ and $^{*}P<0.05$ when compared with TPO-treated cells

the effect was however less pronounced than with DPI (Supplementary Figure 4e), without affecting cell viability (Supplementary Figure 4f) or cell proliferation (Supplementary Figure 4g). Taken together, these experiments show that inhibition of NADPH oxidase activity alters megakaryocytic differentiation, and that this effect cannot be attributed to an indirect effect on cell viability.

This again shows that ROS production is required for megakaryocytic differentiation, and strongly point to one or more NADPH oxidases as being necessary players in differentiation.

To know whether the inhibition of ROS production was halting or delaying cell differentiation, we followed differentiation at different times in the presence or absence of quercetin or DPI. In HEL cells, both treatments seemed to slow down differentiation because the marker levels were significantly lower in the presence of quercetin or DPI (Supplementary Figure 5a and b), but their dynamics seemed to mirror that of

PMA alone. In the case of K562 cells, it is more difficult to draw firm conclusions because although the expression of CD61 was slowed down, the expression of CD41 seemed to be blocked by both treatments (Supplementary Figure 5c and d).

p22^{phox}-dependent NADPH oxidase activity is involved in megakaryocytic differentiation. p22^{phox} is a plasma membrane protein necessary for the integrity of different NADPH complexes to be maintained.[5] Thus, the downregulation of p22^{phox} levels should lead to a decrease in the levels of these enzymes. p22^{phox} was knocked down by RNA interference (RNAi). The four sequences tested decreased p22^{phox} protein levels (Figure 4a). As it could be expected, NADPH oxidase activity was significantly lower when the p22^{phox} protein was downregulated (Figure 4b). This led to the inhibition of differentiation in both cell lines, as the levels of the megakaryocytic markers (CD41 and CD61) were significantly lower and the decrease in the expression in the erythrocytic marker GpA, was also prevented when p22^{phox} protein levels were downregulated (Figure 4c–e). Moreover, p22^{phox} downregulation did not reduce cell viability or proliferation (Supplementary Figure 6).

We next carried out p22^{phox} RNAi experiments in human CD34$^+$ cells using the same target sequences as in cell lines. After 7 days of TPO treatment, almost 80% of the cells were differentiated (Figure 5a). Differentiation was hampered when cells were infected with short hairpin RNA (shRNA) directed against p22^{phox}, because the number of CD41/CD61 double-positive and CD42b-positive cells (Figure 5b) and the levels of all megakaryocytic markers (Figure 5c) were significantly lower in these cells compared with control cells infected with the shRNA directed against luciferase.

These results, together with the results described in Figures 3 and 4, suggest that one or more p22^{phox} NADPH oxidase-dependent complexes would be responsible for ROS production and that they would therefore be required for cell differentiation of both cell lines and human CD34$^+$ cells. This strongly suggests that p22^{phox} NADPH oxidase-driven ROS production must also be important for *in vivo* megakaryocytopoiesis.

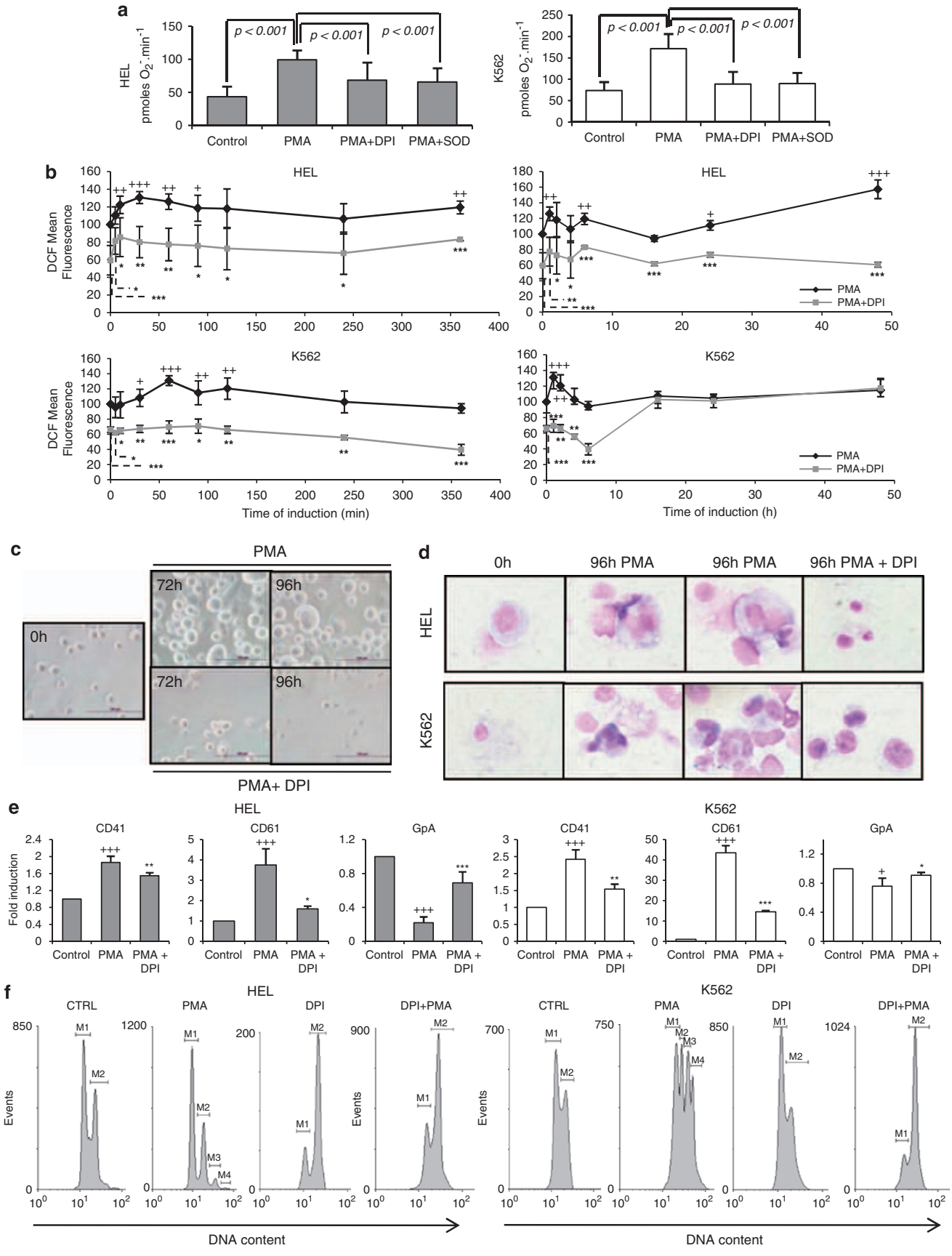
Analysis of signal transduction pathways activated during megakaryocytopoiesis. As ROS could be affecting the signalling pathways that control megakaryocytic differentiation, we first decided to analyse the relevance of the signalling pathways activated during this specific differentiation. We detected the phosphorylation, and consequently the activation, of ERK and c-Jun N-terminal kinase (JNK) in both cell lines, and the activation of AKT, STAT3 and STAT5 transcription factors in HEL cells (Supplementary Figure 7). The contribution of all these pathways to differentiation was analysed using specific inhibitors. Regarding the expression of megakaryocytic markers (Table 1), a significant inhibition of their expression in both cells lines was observed when MEK was inhibited, this effect being more pronounced than with any other inhibitor. AKT inhibition also hindered differentiation. Surprisingly, however, the inhibition of phosphatidylinositol 3-kinase (PI3K) had hardly any effect, suggesting a PI3K-independent activation of AKT. The inhibition of JNK or JAK2 also had some effect, although limited to only some of the markers. Regarding the state of the cells, only the inhibition of AKT diminished the percentage of viable cells, which could in part explain its effect on cell differentiation (Supplementary Figure 8).

In human CD34$^+$ cells, the picture was quite similar (Figure 6): the inhibition of MEK, AKT and JAK significantly decreased marker expression. The inhibition of PI3K seemed to increase CD42b levels significantly, but had no significant effect on CD41 or CD61 levels. Finally, the inhibition of JNK produced a significant increase in all megakaryocytic markers. These effects were also reflected in cell morphology, as the increase in cell size that accompanied differentiation was prevented by the inhibition of MEK and AKT, but not by the inhibition of JNK (Figure 6d).

NADPH oxidase ROS production is required for the full activation of the signalling pathways that lead to megakaryocytic differentiation. Next, we speculated whether these signalling pathways might somehow be affected by ROS. To test this hypothesis, we analysed the activation of these pathways in the presence or absence of DPI. Our data showed that DPI hampered the activation of ERK in K562 (Figure 7a) and HEL cells stimulated with PMA (Figure 7b). DPI also seemed to hamper the weak activation of STAT3 and STAT5 in HEL cells stimulated with PMA (Figure 7b). We then speculated whether this would also hold true for TPO signalling. As shown in Figure 7c, DPI hampered the activation of AKT, STAT3 and STAT5 in HEL cells stimulated with TPO. Finally, we looked at these signalling pathways in cells in which we had knocked down the p22^{phox} protein. In these cells, the activation of ERK, STAT-5 or AKT was significantly lower with respect to control cells (Figure 7d). Quantification analysis showed that the activation of all signalling pathways required for differentiation was significantly lower in the presence of DPI or in cells in which p22^{phox} had been knocked down.

Overall, these results allow us to propose that NADPH oxidase-driven ROS production is required for complete activation of all signalling pathways needed for megakaryocytic

Figure 3 NADPH oxidase activity is required for megakaryocytic differentiation of K562 and HEL cells. (**a**) NADPH oxidase activity measured by extracellular superoxide (O_2^-) production in HEL and K562 cells in response to 20 μM PMA in the presence or absence of 50 Units SOD or 50 μM DPI (means ± S.D. of three different experiments performed in triplicate). HEL and K562 cells were differentiated with 20 nM PMA in the presence or absence of 5 μM DPI, and the following parameters were analysed: (**b**) Intracellular ROS production was measured by flow cytometry cells labelled with DCFDA. ROS levels in response to PMA or PMA plus DPI were normalised to those of untreated cells; (**c**) microphotograph of HEL cells treated with PMA alone or PMA plus DPI for 72 and 96 h; (**d**) cell staining with May–Grünwald–Giemsa of cells differentiated during 96 h in the presence or absence of DPI; (**e**) megakaryocytic marker expression was analysed by flow cytometry in cells differentiated during 48 h in the presence or absence of DPI; the marker levels (means ± S.D. of four different experiments) are shown; (**f**) polyploid cells were identified by PI staining after 48 h of differentiation. $^{+++}P<0.001$, $^{++}P<0.01$ and $^{+}P<0.05$ when compared with PMA-untreated control cells; $^{***}P<0.001$, $^{**}P<0.01$ and $^{*}P<0.05$ when compared with PMA-treated cells


a
HEL
pmoles O_2^-.min^{-1}
p < 0.001
p < 0.001
p < 0.001
Control
PMA
PMA+DPI
PMA+SOD
K562
b
HEL
K562
DCF Mean Fluorescence
PMA
PMA+DPI
Time of induction (min)
Time of induction (h)
c
PMA
0h
72h
96h
PMA+ DPI
d
0h
96h PMA
96h PMA
96h PMA + DPI
HEL
K562
e
HEL
K562
CD41
CD61
GpA
Fold induction
Control
PMA
PMA + DPI
f
HEL
K562
CTRL
PMA
DPI
DPI+PMA
Events
DNA content

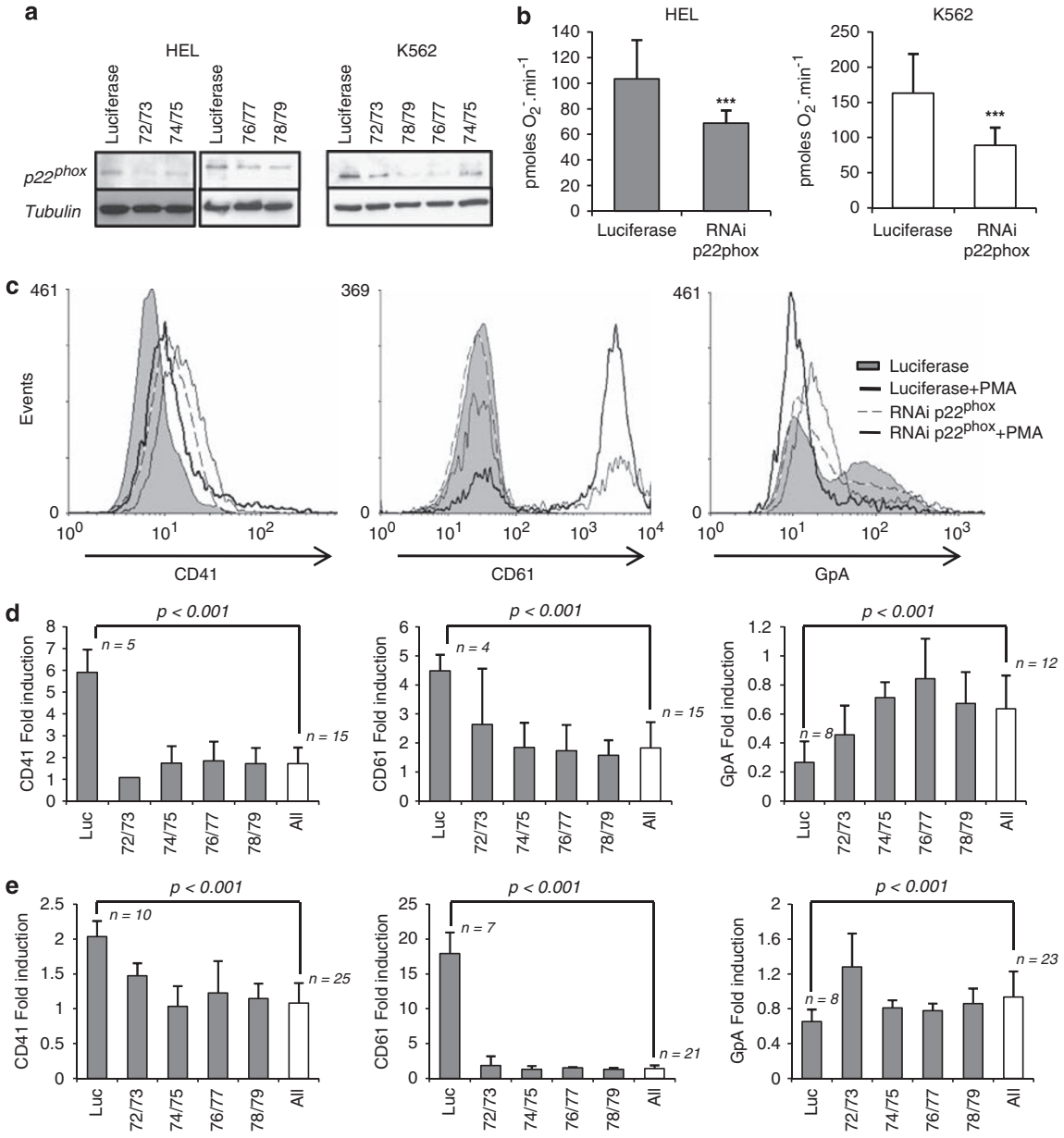


Figure 4 A p22^{phox}-dependent NADPH oxidase activity is required for megakaryocytic differentiation of K562 and HEL cells. p22^{phox} expression was downregulated by RNAi experiments in HEL and K562 cells. Four different p22^{phox} target sequences were used (namely 72/73, 74/75, 76/77 and 78/79), and a sequence directed against firefly luciferase was used as a control. (**a**) Immunoblot showing that the four shRNAs used were able to reduce p22^{phox} levels in HEL and K562 cells. (**b**) NADPH oxidase activity measured by extracellular superoxide (O_2^-) production in HEL and K562 cells subjected to RNAi experiments in response to 20 μM PMA (means ± S.D. of three different experiments performed in triplicate, $^{***}P < 0.001$). The different cells were subjected to megakaryocytic differentiation by PMA treatment over 48 h, and the megakaryocytic marker expression (CD41, CD61 and GpA) was analysed. (**c**) A representative experiment in K562 cells. Fold increase in megakaryocytic marker levels after PMA treatment of HEL (**d**) and K562 cells (**e**). The results are means ± S.D. The column in white represents the means ± S.D. of all RNAi experiments

differentiation. Thus, ROS would be acting upstream of all these signalling pathways.

Discussion

The role of ROS as second messengers, the so-called 'redox signalling', has been a recurrent theme in the field of signalling during the last few years. ROS production has mainly been related to cell growth,[23] with only a few reports addressing their involvement in the triggering of specific differentiation programmes being available.[6,7,24] Although ROS production in response to haematopoietic cytokines has long been known,[10] little is known about their possible involvement in haematopoiesis. Accordingly, we were therefore prompted to study this issue in depth using megakaryocytic differentiation as a system.

In this study, we show that ROS formation is required for megakaryocytic differentiation. ROS formation was very rapid, in agreement with previous reports[25] and was prevented by antioxidant treatment, which also hindered

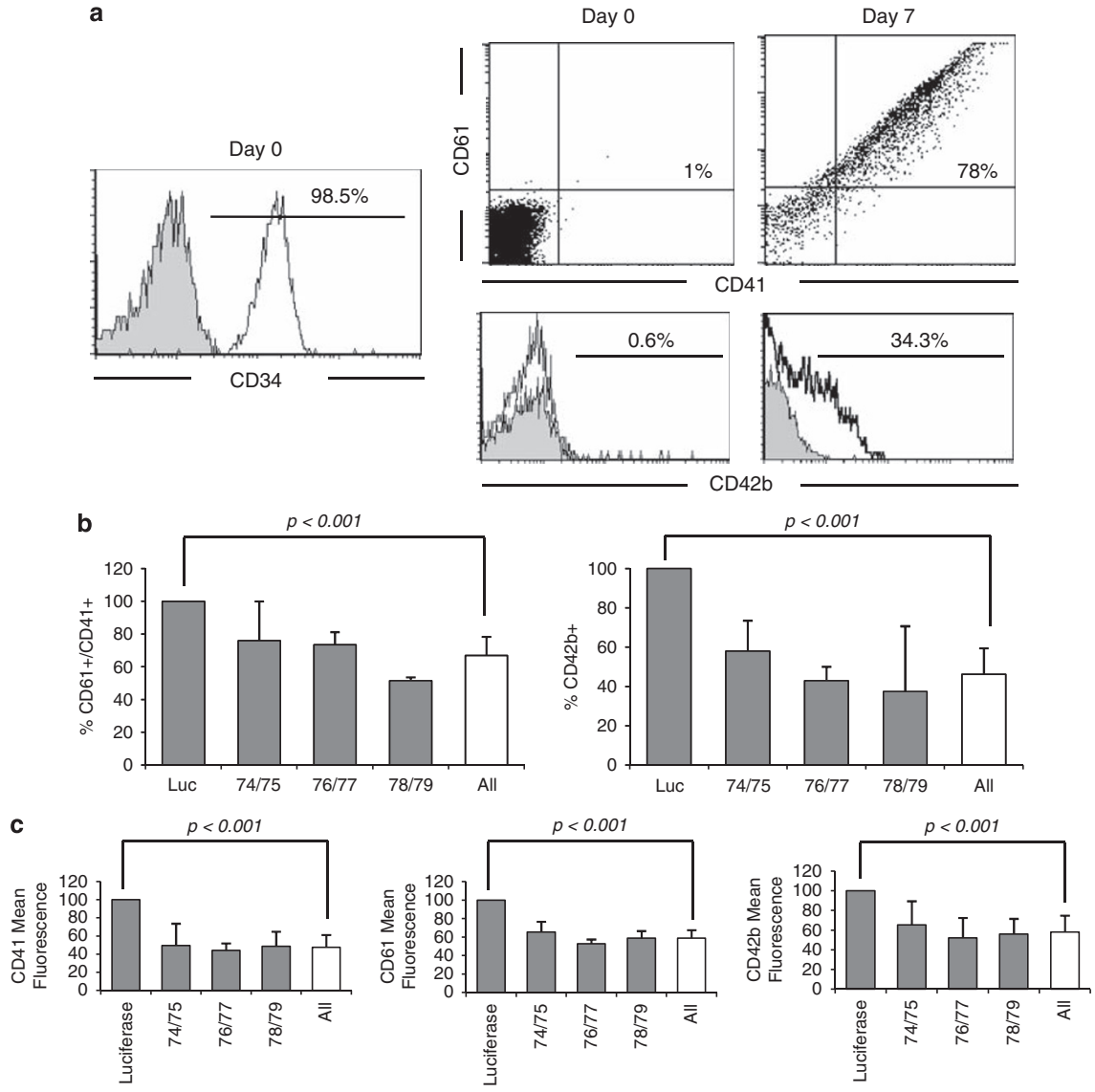


Figure 5 A p22^{phox}-dependent NADPH oxidase activity is required for megakaryocytic differentiation of human CD34$^+$ cells. Human CD34$^+$ cells isolated from umbilical cord blood cultured in the Stem Cell Growth Medium plus 100 ng/ml TPO. (**a**) CD34$^+$ cell purity was >98% after cell sorting (day 0); CD41, CD61 and CD42b protein expression was analysed at day 0; megakaryocytic differentiation was checked after 7 days of culture in the presence of TPO (100 ng/ml). A representative experiment is shown. (**b**) CD34$^+$ cells were infected with lentivirus carrying an H1 promoter-shRNA cassette and GFP as a marker. Three different p22^{phox} target sequences (namely 74/75, 76/77 and 78/79) and a sequence directed against firefly luciferase were used as a control. Percentages of cell differentiation (CD42b-positive cells and CD41/CD61-double-positive cells) in GFP$^+$ cells of every single shRNA relative to the control (Luc GFP$^+$-infected cells) are shown. (**c**) Megakaryocytic marker levels were lower in cells infected with p22^{phox} shRNA target sequences (74/75, 76/77 and 78/79) than in control cells. Results show the mean ± S.D. of two independent experiments with each target sequence

differentiation. The involvement of ROS was observed for both cell lines and primary cells studied, so it is also likely to occur *in vivo*.

Different experimental approaches, such as the measurement of NADPH oxidase activity, the use of NADPH oxidases inhibitors and p22^{phox} RNAi experiments, showed that ROS production is a consequence of a p22^{phox}-dependent NADPH oxidase. In this sense, it should be noted that NADPH oxidases seem to be involved in ROS formation in response to granulocyte colony-stimulating factor (G-CSF).[23] Therefore, we suggest that NADPH oxidase ROS production might be a mechanism common to all haematopoietic cytokines. Interesting issues in the future will be the identification of the specific NADPH oxidase(s) involved in megakaryocytic differentiation. It would also be interesting to analyse whether different haematopoietic cytokines function through specific NADPH oxidases. In this sense, it is known that HSCs express different Nox family members,[26] whose particular roles are unknown, although according to our results, it is possible that they could participate in the regulation of haematopoiesis.

Whichever the case, our results clearly show that NADPH oxidase activity is crucial for the redox signalling cascade that leads to megakaryocytic differentiation to be initiated.

TPO, probably the most important cytokine for megakaryocytopoiesis,[27] activates different signalling pathways (such as JAK2/STAT, PI3K/AKT, MEK/ERK and JNK pathways).[28] It has previously been proposed that the activation of ERK1/2 and JNK, together with the inhibition of p38 MAPK, would be crucial for K562 cell megakaryocytic differentiation.[29] In this study, we analysed the signalling pathways that are important for megakaryocytic differentiation not only in K562 but also in HEL cells and human $CD34^+$ cells. According to our results, the inhibition of MEK, AKT and JAK2 prevented differentiation in all types of cells. The inhibition of JNK only had small effects in HEL cell differentiation, and the inhibition of PI3K had little effect on differentiation. The importance of MAPKs for megakaryocytopoiesis has long been known,[30] so our data would support this. Previous reports have suggested that the PI3K/AKT pathway is not necessary for the differentiation of K562 cells,[29] although other authors have related this pathway to endomitosis in $CD34^+$ cells.[27] In this study, we show that whereas PI3K had no effect on differentiation, AKT inhibition strongly inhibited it, and that these features were consistent in all types of cells studied. Therefore, it is likely that AKT would be activated through a mechanism not dependent on PI3K.

It is remarkable that the transient ROS burst proved to be so crucial for full activation of the signalling cascades and the triggering of the differentiation programme to be achieved. Our results support this idea, because a PMA treatment of only 2 h—time enough for the ROS burst to occur—is enough to induce cell differentiation. The ROS burst would be probably one of the first events during megakaryocytopoiesis, and according to our results, ROS would be upstream of all the signalling cascades required for differentiation. Nevertheless, the existence of a feed-forward loop is also possible, that is, the assembly of the NADPH oxidase involved in differentiation might be under the control of some of the signalling pathways activated during the process, and the ensuing ROS production would have a positive effect leading to full activation of the signalling cascades. In this sense, it is important to note that NADPH oxidase complex assembly is dependent on $p47^{phox}$ phosphorylation,[5] which occurs in response to several different kinases,[31] some of which are important for megakaryocytopoiesis.

The activation of different signalling pathways in response to ROS has been described previously,[25] but the molecular mechanism underlying this observation is not completely understood. ROS seem to activate signalling cascades depending on the context, that is, ROS production in response to G-CSF would be related to AKT activation but not to ERK activation,[23] whereas in our system, ROS were involved in the activation of both pathways. Exactly how redox signalling produces these specific effects is an interesting question. One possibility would be that the different compartmentalisation of NADPH oxidases[32] might activate different signals. Another possibility is that the molecular targets of ROS may differ, depending on the scenario. In our context, a key question for future studies would be to determine the mechanism by which ROS activate the signalling pathways required for megakaryocytic differentiation. One of the most widespread suggestions about the mechanism of action of ROS is the inhibition of the phosphatases that regulate these signalling pathways. In this regard, it has recently been shown that the inhibition of PP1α by ROS leads to the constitutive activation of the PI3K/AKT pathway in leukaemic cells.[33]

In addition, it has been shown that ROS are also important for platelet release,[34] this points to the importance of ROS not only in triggering differentiation programmes but also in terminal platelet release from mature megakaryocytes.

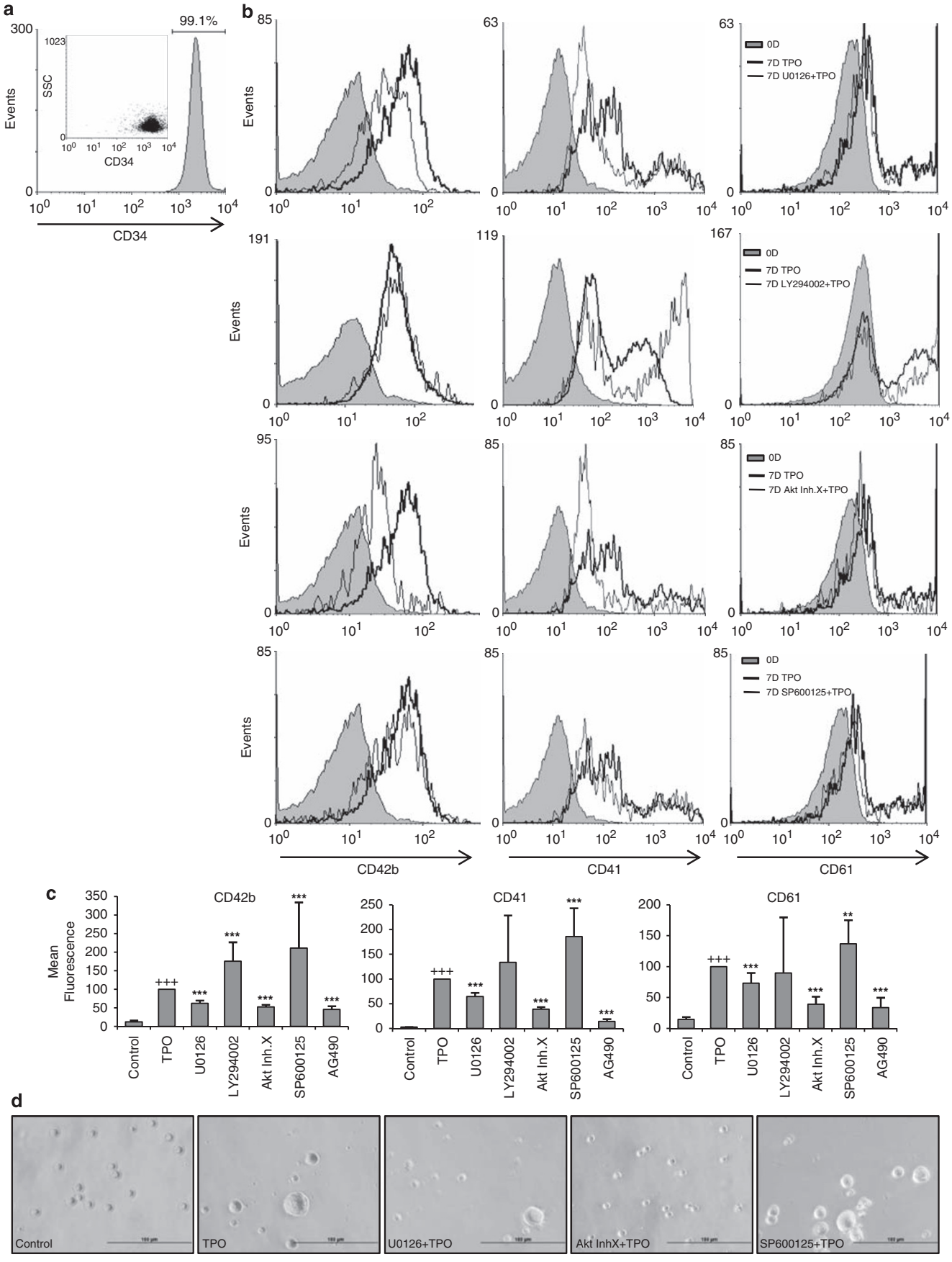
In summary, in this study, we showed for the first time that ROS production is required for megakaryocytic differentiation.

Table 1 Effect of different signalling pathway inhibitors on megakaryocytic marker expression in K562 and HEL cells

	K562				HEL			
Treatment	**CD42b**	**CD41**	**CD61**	**GpA**	**CD42b**	**CD41**	**CD61**	**GpA**
PMA	$1.55 \pm 0.05^{+++}$	$2.15 \pm 0.32^{+++}$	$61.12 \pm 4.30^{+++}$	$0.64 \pm 0.07^{+++}$	$2.18 \pm 0.06^{+++}$	$2.10 \pm 0.27^{+++}$	$4.09 \pm 0.41^{+++}$	$0.33 \pm 0.10^{+++}$
U0126+PMA	1.04 ± 0.51	$0.99 \pm 0.06^{***}$	$3.50 \pm 1.44^{***}$	0.49 ± 0.17	$0.84 \pm 0.01^{***}$	$1.09 \pm 0.11^{***}$	$3.21 \pm 0.21^{**}$	$0.71 \pm 0.19^{*}$
LY294002+PMA	1.52 ± 0.03	1.89 ± 0.17	34.50 ± 29.59	$1.49 \pm 0.02^{***}$	1.88 ± 0.01	2.27 ± 0.55	3.72 ± 0.49	0.27 ± 0.05
AKT Inh.X+PMA	1.09 ± 0.27	$1.55 \pm 0.21^{*}$	$14.65 \pm 1.12^{***}$	$1.15 \pm 0.12^{***}$	$1.69 \pm 0.01^{***}$	$1.39 \pm 0.21^{**}$	$1.48 \pm 0.11^{***}$	0.28 ± 0.06
SP600125+PMA	1.12 ± 0.07	1.87 ± 0.26	$31.18 \pm 19.61^{*}$	$1.98 \pm 0.08^{***}$	2.17 ± 0.04	1.59 ± 0.38	$3.08 \pm 0.03^{**}$	$0.78 \pm 0.18^{**}$
AG490+PMA	1.35 ± 0.13	$1.32 \pm 0.05^{**}$	$5.38 \pm 1.49^{***}$	0.62 ± 0.33	1.84 ± 0.32	2.08 ± 0.04	$1.93 \pm 0.35^{***}$	$0.72 \pm 0.12^{**}$

Cells were treated with 20 nM PMA for 48 h in the presence or absence of 10 μM U0126 (MEK inhibitor), 10 μM LY294002 (PI3K inhibitor), 10 μM AKT Inhibitor X, 10 μM SP600125 (JNK inhibitor) or 50 μM AG490 (JAK inhibitor). The results show the megakaryocytic markers (CD42b, CD41 and CD61) or erythrocytic marker (GpA) fold increase with respect to control cells. For PMA-differentiated cells, control cells were treated with vehicle (DMSO) diluted 10^5 times. For PMA+inhibitor treatment, control cells were treated with DMSO+inhibitor. The means ± S.D. of four different experiments are shown. $^{+++}P<0.001$, reflects significant differences of PMA-treated cells with respect to undifferentiated cells. $^{*}P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$ reflect significant differences of different inhibitor treatments when compared with PMA-treated cells

Figure 6 Analysis of signalling pathways involved in megakaryocytic differentiation of human $CD34^+$ cells. Human $CD34^+$ cells were subjected to differentiation by treatment with 100 ng/ml TPO in the presence or absence of specific inhibitors for MEK (10 μM U0126), JNK (10 μM SP600125), PI3K (10 μM LY294002), AKT (10 μM AKT Inhibitor X) and JAK2 (50 μM AG490). (**a**) $CD34^+$ cell purity was >99%. (**b**) A representative experiment showing the effect of these inhibitors on the expression of differentiation markers. (**c**) Megakaryocytic marker levels (means ± S.D. of four different experiments) are shown. $^{+++}P<0.001$ when compared with undifferentiated control cells; $^{***}P<0.001$ and $^{**}P<0.01$ when compared with TPO-treated cells. (**d**) Inhibition of AKT and MEK, but not JNK, prevented the increase in cell size accompanying differentiation


a
99.1%
Events
SSC
CD34
b
0D
7D TPO
7D U0126+TPO
7D LY294002+TPO
7D Akt Inh.X+TPO
7D SP600125+TPO
CD42b
CD41
CD61
c
Mean Fluorescence
Control
TPO
U0126
LY294002
Akt Inh.X
SP600125
AG490
d
Control
TPO
U0126+TPO
Akt InhX+TPO
SP600125+TPO

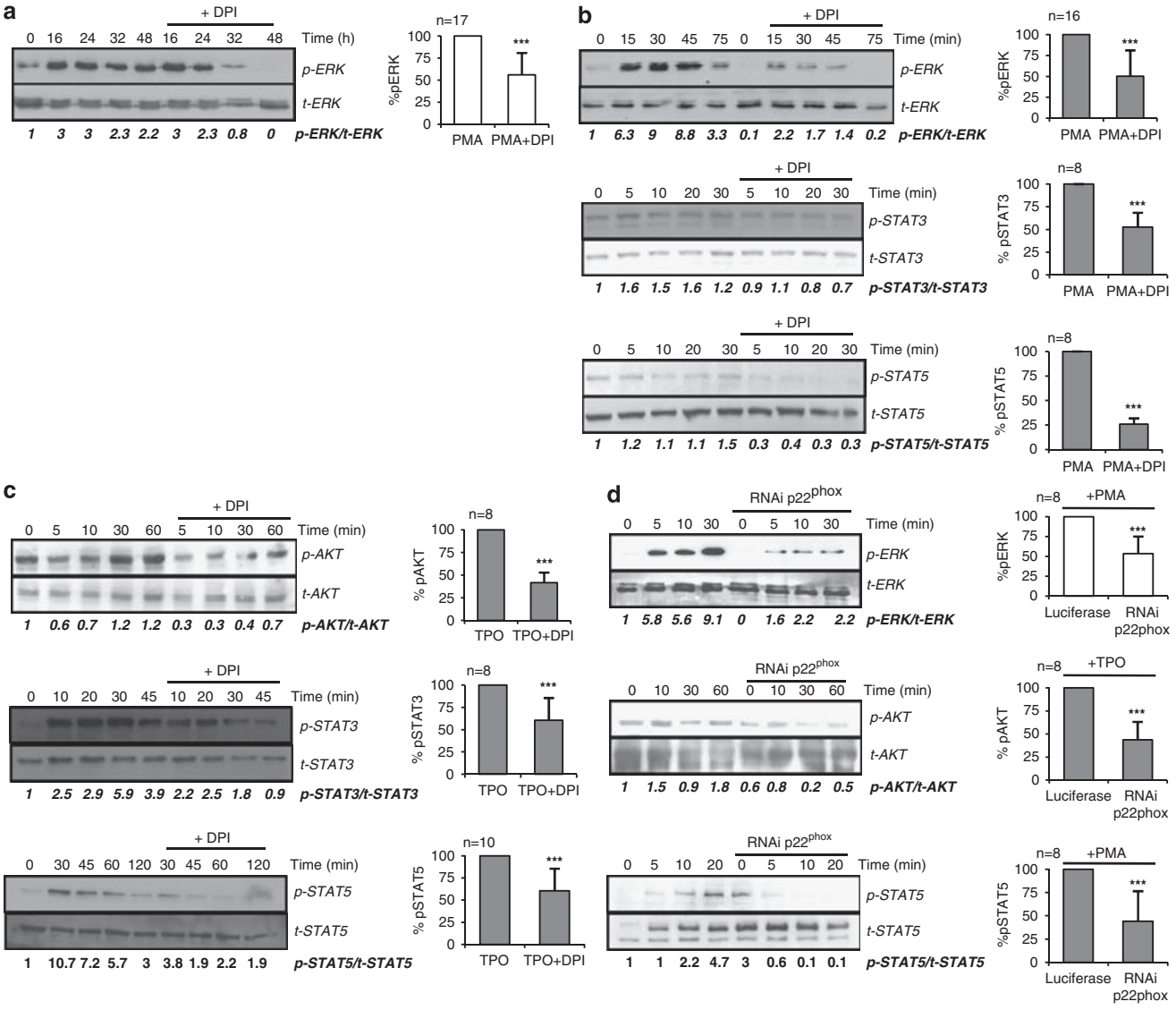


Figure 7 ROS production is required for activation of the signalling pathways that control megakaryocytic differentiation. Activation of signalling cascades was analysed by immunoblotting with antibodies that recognised the phosphorylated/active forms of ERK, AKT, STAT3 and STAT5. The same membranes were stripped and reprobed to check the total levels of these proteins. To know whether NADPH oxidase ROS production was important for the activation of these signalling cascades, their activation was analysed in the presence or absence of DPI, and in cells in which the $p22^{phox}$ RNAi had been performed. The levels of the phosphorylated/active forms were quantified and normalised with respect to the total levels of these proteins; numbers are depicted below the blots. The histograms represent the percentage of activation reached under different experimental conditions (means ± S.D.; n = number of different samples analysed coming from between two and four independent experiments, $^{***}P<0.001$). (**a**) Levels of phosphorylated ERK in K562 cells stimulated with 20 nM PMA in the presence or absence of 5 μM DPI. (**b**) Levels of the phosphorylated forms of ERK, STAT3 and STAT5 in HEL cells stimulated with 20 nM PMA in the presence or absence of 5 μM DPI. (**c**) Levels of the phosphorylated forms of AKT, STAT3 and STAT5 in HEL cells stimulated with 100 ng/ml TPO in the presence or absence of 5 μM DPI. (**d**) Signalling pathway activation in cells in which the $p22^{phox}$ protein had been downregulated was impaired with respect to control cells. A representative experiment is depicted showing the activation of ERK in K562 cells and the activation of STAT5 and AKT in HEL cells

A $p22^{phox}$-dependent NADPH oxidase activity is responsible for the ROS burst, leading to full activation of the signalling cascades required for differentiation. All the results obtained in this study hold true for cell lines and primary cells, and hence we propose that our observations may also be relevant for *in vivo* megakaryocytopoiesis. It will be interesting to identify the particular NADPH oxidase involved in this process and also to elucidate how ROS activate the signalling cascades. These questions will be addressed in future studies.

Materials and Methods

Reagents. RPMI medium, fetal bovine serum (FBS), penicillin, streptomycin and L-glutamine were acquired from Lonza (Barcelona, Spain). TPO and Stem Cell Growth Medium were obtained from Cellgenix GmbH (Freiburg, Germany). PMA, 2′,7′-dichlorofluorescein diacetate (DCFDA), propidium iodide (PI), dimethyl sulphoxide (DMSO), DPI, NAC, quercetin, Trolox, chloroquine, Top-Block, anti-β-tubulin and the selective inhibitor of JNK SP600125, cytochrome *c*, NADPH, superoxide dismutase (SOD), MTT, TMRE were from Sigma-Aldrich (Madrid, Spain). PI3K-specific inhibitor LY294002, AKT inhibitor X, JAK2 selective inhibitor AG490 and G418 sulphate were purchased from Calbiochem (Darmstadt, Germany). MEK inhibitor U0126 was from Promega Biotech Iberica (Madrid, Spain). Flow cytometry

antibodies (CD42b-FITC (CD42b-fluorescein isothiocyanate), CD42b-PerCP (CD42b-peridinin chlorophyll protein), CD41-PE (CD41-phycoerythrin), CD61-APC (CD61-allophycocyanin), GpA-PE) were obtained from Inmunostep (Salamanca, Spain). Antibodies against p-AKT (Ser 473), p-SAPK/JNK (Thr183/Tyr185), p-Stat3 (Tyr 705) and p-Stat5 (Tyr 694) were acquired from Cell Signalling Technology (Danvers, MA, USA). Antibodies against p-ERK (E-4), ERK 1 (K-23), AKT 1/2/3 (H-136), JNK1 (FL), Stat3 (F-2), Stat5 (H-134) and $p22^{phox}$ (C-17) were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Polyvinylidene fluoride (PVDF) membranes and ECL-Plus reagent were obtained from GE Healthcare (Barcelona, Spain). $CD34^+$ Microbead Kit was from Miltenyi Biotec (Madrid, Spain). FACS lysing solution and apoptosis detection kit were from BD Bioscience (Madrid, Spain). Retronectin was from Takara (Saint-Germain-en-Laye, France).

Cell lines. The human erythroleukaemic cell lines K562 and HEL were grown in RPMI medium supplemented with 10% FBS, 100 Units/ml penicillin, 100 Units/ml streptomycin and 2 mM L-glutamine. Cells (3×10^5 cells per ml) were treated with 20 nM PMA to induce megakaryocytic differentiation. With the exception of 50 μM AG490, which was added 60 min previously, all antioxidants or specific inhibitors were added 30 min before PMA at the following concentrations: 100 μM NAC, 100 μM quercetin, 10 μM Trolox, 5 μM DPI, 10 μM U0126, 10 μM SP600125, 10 μM LY294002 and 10 μM AKT Inhibitor X.

Human $CD34^+$ purification. Human $CD34^+$ cells were purified from adult peripheral blood volunteer donors or from umbilical cord blood. Low-density mononuclear cells were isolated by Ficoll–Hypaque density-gradient centrifugation, and $CD34^+$ cells were then purified from Miltenyi MACS columns, according to the manufacturer's instructions. Cell purity, evaluated by flow cytometry with CD34 antibody, was $\sim$98%. Cells were resuspended in the Stem Cell Growth Medium (1×10^5 cells per ml) and subjected to megakaryocytic differentiation by treatment with 100 ng/ml TPO during 7 days. Approval was obtained from the Institutional Review Board of the University Hospital of Salamanca for these studies, and informed consent for volunteer donors was provided according to the Declaration of Helsinki.

Differentiation analyses. Megakaryocytic differentiation was followed by the increase in megakaryocytic markers (CD42b, CD41 and CD61) and the decrease in the erythrocytic marker GpA. Cells (5×10^4) were centrifuged, resuspended in 50 μl of PBS and incubated 15 min at room temperature (in the darkness) after adding 5 μl of the corresponding antibody. Cells were then washed and resuspended in 200 μl of PBS. To determine DNA contents, cells were washed in PBS, fixed with 1 : 10 FACS lysing solution, permeabilised with 0.05% Triton X-100 and finally stained with 50 μg/ml PI. Samples were acquired using a FACScalibur Flow Cytometer using the CellQuest Pro Program (BD Bioscience). The results were analysed using the WinMDI 2.9 Program (Joseph Trutter, Purdue University, West Lafayette, IN, USA). The increase in cell size was examined under an Olympus IX51 light microscope (Olympus America, Inc., Melville, NY, USA) with a $\times$40 objective. Cells were also stained with May–Grünwald–Giemsa to visualise the morphological features of megakaryocytes, that is, larger cells, with polylobulated or polysegmented nuclei and a basophilic cytoplasm.

Measurement of intracellular ROS levels. The cell-permeant probe, DCFDA, which shows fluorescence when it is oxidised, was used to measure intracellular ROS. Cells were incubated with 10 μM DCFDA in RPMI plus 1% FBS at 37 °C for 30 min, after which they were washed twice with PBS, resuspended in fresh complete medium and finally treated with 20 nM PMA. ROS production was measured by flow cytometry.

Measurement of extracellular O_2^- production. NADPH oxidase activity was followed by extracellular O_2^- production, that was measured by the SOD-inhibitable reduction of cytochrome *c*. Briefly, 2.5×10^5 cells were resuspended in 200 μl of PBS containing 250 μM cytochrome *c* and 100 μM NADPH and stimulated with different concentrations of PMA in the presence or absence of 50 Units of SOD or 50 μM DPI. Cytochrome *c* reduction was recorded at 37 °C in an ELISA reader at 550 nm. The amount of O_2^- released was calculated using an extinction coefficient of 21 $mM^{-1}\ cm^{-1}$.

Immunoblotting. Cells resuspended in MLB lysis buffer (25 mM HEPES, pH 7.5, 150 mM NaCl, 1% Igepal, 10% glycerol, 10 mM $MgCl_2$, 1 mM EDTA, 25 mM NaF, 1 mM Na_2VO_4, plus proteinase inhibitors) were incubated on ice for 20 min, and non-soluble material was eliminated by centrifugation. Protein concentrations were determined using the Bradford assay. The samples were then subjected to SDS-PAGE and the proteins were transferred onto PVDF membranes, as described previously.[35] Non-specific binding was blocked with 5% non-fat dry milk or 5% Top-Block. Incubation with primary antibodies at the appropriate dilution was performed overnight at 4 °C. Secondary antibody incubation was performed at room temperature for 1 h. Blots were visualised using chemiluminescence using ECL-Plus reagent.

$p22^{phox}$ RNAi. $p22^{phox}$ levels were downregulated by RNAi using the pSUPER-neo/GFP vector carrying the Pol III-dependent H1 promoter.[36] An oligonucleotide against firefly luciferase was used as a control.[36] Four different $p22^{phox}$ target sequences were used: 5′-CCATGTGGGCCAACGAACA-3′, 5′-ACATGACCGCCG TGGTGAA-3′, 5′-TACTTTGGTGCCTACTCCA-3′ and 5′-AGATCGGAGGCACCAT CAA-3′. In all, 10^7 cells were transfected with 25 μg of DNA by electroporation using a Bio-Rad Gene Pulser (Bio-Rad Laboratories, Barcelona, Spain; 300 V, 60 Ohms, 960 μF). Transfected cells were selected by treatment with G418 for 2 weeks (0.25 mg/ml for K562 and 0.125 mg/ml for HEL cells). Human $CD34^+$ cells RNAi was performed as described before.[37] The H1-shRNA expression cassette was excised from pSUPER vector and cloned in the lentiviral pLVTHM plasmid using EcoRI–ClaI sites. Viral particles were produced in human embryonic kidney 293T cells seeded in high-glucose DMEM containing 10% FBS. pRRE, pREV, phCMV-GALV-TR and the lentiviral vector pLVTHM containing both the GFP reporter gene and the shRNA sequence were transfected in the packaging cell line by calcium phosphate precipitation in the presence of 25 μM chloroquine. Human $CD34^+$ cells were seeded in 12-well retronectin-coated plates (5 $\mu g/cm^2$) in which GALV-TR-pseudotyped lentiviral vectors had been preloaded and were cultured in the Stem Cell Growth Medium plus 100 ng/ml TPO. The mean multiplicity of infection, determined using HT1 cells, was $\sim$10 infectious particles per target cell. After 24 h, cells were transferred to a new well with fresh medium. GFP expression was analysed by FACS analysis. Differentiation analyses were carried out on day 7.

Cell viability analysis. Cell cultures and differentiation were performed as described above. Cell viability was determined by the Annexin V-PE/7-aminoactinomycin (7-AAD) apoptosis detection kit from BD Bioscience. Briefly, 5×10^5 cells were washed and resuspended in the binding buffer (1 : 10 diluted in H_2O). A volume of 5 μl of Annexin V-PE and 5 μl 7-AAD were added and incubated for 15 min. For every condition, 50 000 events were collected and analysed. The percentage of viable cells (Annexin V-PE/7-AAD double-negative cells) was calculated using the WinMDI 2.9 Program.

MTT proliferation assay. Cell proliferation was studied by the MTT assay (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide). Cells cultured and differentiated as described above were washed with PBS and then resuspended in PBS containing 0.5 mg/ml MTT and incubated at 37 °C for 75 min. Cells were then washed in PBS and finally resuspended in a suitable volume of DMSO. Absorbance was measured at 570 nm.

Mitochondrial potential assay. Mitochondrial potential was measured by the accumulation of TMRE (tetramethyl rhodamine ethyl ester). Cells cultured and differentiated as described above, were incubated with 10 nM TMRE in RPMI plus 1% FBS at 37 °C for 30 min, after which they were washed twice with PBS, and resuspended in PBS. TMRE fluorescence was measured by flow cytometry.

Statistical analyses. Data are expressed as means $\pm$ S.D. Statistical analyses were performed using Student's *t*-test. Differences were considered statistically significant when $P < 0.05$.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements. This work was supported by MCINN (BFU2006-10362, BFU2009-10568 and CSD2007-00015), by JCyL (SA010A10-2 and SA126A071) and FIS (PS09/01075). JLS was a recipient of a predoctoral fellowship from the *Regional Government of Castile and León*, Spain. We thank Dr. C Calés for providing us with HEL cells and Dr. JP Bolaños for the pSuperLuc plasmid. We thank Dr. M Díez Campelo for her assistance with the morphological analysis. We also thank N Skinner for reviewing the English version of this paper.

1. Genestra M. Oxyl radicals, redox-sensitive signalling cascades and antioxidants. *Cell Signal* 2007; **19**: 1807–1819.
2. Tatla S, Woodhead V, Foreman JC, Chain BM. The role of reactive oxygen species in triggering proliferation and IL-2 secretion in T cells. *Free Radic Biol Med* 1999; **26**: 14–24.
3. Goldstone SD, Milligan AD, Hunt NH. Oxidative signalling and gene expression during lymphocyte activation. *Biochim Biophys Acta* 1996; **1314**: 175–182.
4. Nauseef WM. Biological roles for the NOX family NADPH oxidases. *J Biol Chem* 2008; **283**: 16961–16965.
5. Bedard K, Krause KH. The NOX family of ROS-generating NADPH oxidases: physiology and pathophysiology. *Physiol Rev* 2007; **87**: 245–313.
6. Buggisch M, Ateghang B, Ruhe C, Strobel C, Lange S, Wartenberg M *et al.* Stimulation of ES-cell-derived cardiomyogenesis and neonatal cardiac cell proliferation by reactive oxygen species and NADPH oxidase. *J Cell Sci* 2007; **120**: 885–894.
7. Tsatmali M, Walcott EC, Makarenkova H, Crossin KL. Reactive oxygen species modulate the differentiation of neurons in clonal cortical cultures. *Mol Cell Neurosci* 2006; **33**: 345–357.
8. Ito K, Hirao A, Arai F, Takubo K, Matsuoka S, Miyamoto K *et al.* Reactive oxygen species act through p38 MAPK to limit the lifespan of hematopoietic stem cells. *Nat Med* 2006; **12**: 446–451.
9. Jang YY, Sharkis SJ. A low level of reactive oxygen species selects for primitive hematopoietic stem cells that may reside in the low-oxygenic niche. *Blood* 2007; **110**: 3056–3063.
10. Sattler M, Winkler T, Verma S, Byrne CH, Shrikhande G, Salgia R *et al.* Hematopoietic growth factors signal through the formation of reactive oxygen species. *Blood* 1999; **93**: 2928–2935.
11. Silva A, Yunes JA, Cardoso BA, Martins LR, Jotta PY, Abecasis M *et al.* PTEN posttranslational inactivation and hyperactivation of the PI3K/Akt pathway sustain primary T cell leukemia viability. *J Clin Invest* 2008; **118**: 3762–3774.
12. Yamamoto T, Sakaguchi N, Hachiya M, Nakayama F, Yamakawa M, Akashi M. Role of catalase in monocytic differentiation of U937 cells by TPA: hydrogen peroxide as a second messenger. *Leukemia* 2009; **23**: 761–769.
13. Szalai G, LaRue AC, Watson DK. Molecular mechanisms of megakaryopoiesis. *Cell Mol Life Sci* 2006; **63**: 2460–2476.
14. Tetteroo PA, Massaro F, Mulder A, Schreuder-van Gelder R, dem Borne AE. Megakaryoblastic differentiation of proerythroblastic K562 cell-line cells. *Leuk Res* 1984; **8**: 197–206.
15. Weich HA, Herbst D, Schairer HU, Hoppe J. Platelet-derived growth factor. Phorbol ester induces the expression of the B-chain but not of the A-chain in HEL cells. *FEBS Lett* 1987; **213**: 89–94.
16. Traore K, Sharma RB, Burek CL, Trush MA. Role of ROS and MAPK in TPA-induced ICAM-1 expression in the myeloid ML-1 cell line. *J Cell Biochem* 2007; **100**: 1010–1021.
17. de Mendez I, Leto TL. Functional reconstitution of the phagocyte NADPH oxidase by transfection of its multiple components in a heterologous system. *Blood* 1995; **85**: 1104–1110.
18. van Acker SA, Koymans LM, Bast A. Molecular pharmacology of vitamin E: structural aspects of antioxidant activity. *Free Radic Biol Med* 1993; **15**: 311–328.
19. Gitika B, Sai RM, Sharma SK, Ilavazhagan G, Banerjee PK. Quercetin protects C6 glial cells from oxidative stress induced by tertiary-butylhydroperoxide. *Free Radic Res* 2006; **40**: 95–102.
20. Huber WW, Parzefall W. Thiols and the chemoprevention of cancer. *Curr Opin Pharmacol* 2007; **7**: 404–409.
21. Yamaura M, Mitsushita J, Furuta S, Kiniwa Y, Ashida A, Goto Y *et al.* NADPH oxidase 4 contributes to transformation phenotype of melanoma cells by regulating G2-M cell cycle progression. *Cancer Res* 2009; **69**: 2647–2654.
22. McCrann DJ, Eliades A, Makitalo M, Matsuno K, Ravid K. Differential expression of NADPH oxidases in megakaryocytes and their role in polyploidy. *Blood* 2009; **114**: 1243–1249.
23. Zhu QS, Xia L, Mills GB, Lowell CA, Touw IP, Corey SJ. G-CSF induced reactive oxygen species involves Lyn-PI3-kinase-Akt and contributes to myeloid cell growth. *Blood* 2006; **107**: 1847–1856.
24. Lee NK, Choi YG, Baik JY, Han SY, Jeong DW, Bae YS *et al.* A crucial role for reactive oxygen species in RANKL-induced osteoclast differentiation. *Blood* 2005; **106**: 852–859.
25. Iiyama M, Kakihana K, Kurosu T, Miura O. Reactive oxygen species generated by hematopoietic cytokines play roles in activation of receptor-mediated signaling and in cell cycle progression. *Cell Signal* 2006; **18**: 174–182.
26. Piccoli C, D'Aprile A, Ripoli M, Scrima R, Lecce L, Boffoli D *et al.* Bone-marrow derived hematopoietic stem/progenitor cells express multiple isoforms of NADPH oxidase and produce constitutively reactive oxygen species. *Biochem Biophys Res Commun* 2007; **353**: 965–972.
27. Guerriero R, Parolini I, Testa U, Samoggia P, Petrucci E, Sargiacomo M *et al.* Inhibition of TPO-induced MEK or mTOR activity induces opposite effects on the ploidy of human differentiating megakaryocytes. *J Cell Sci* 2006; **119**: 744–752.
28. Abe M, Suzuki K, Inagaki O, Sassa S, Shikama H. A novel MPL point mutation resulting in thrombopoietin-independent activation. *Leukemia* 2002; **16**: 1500–1506.
29. Jacquel A, Herrant M, Defamie V, Belhacene N, Colosetti P, Marchetti S *et al.* A survey of the signaling pathways involved in megakaryocytic differentiation of the human K562 leukemia cell line by molecular and c-DNA array analysis. *Oncogene* 2006; **25**: 781–794.
30. Whalen AM, Galasinski SC, Shapiro PS, Nahreini TS, Ahn NG. Megakaryocytic differentiation induced by constitutive activation of mitogen-activated protein kinase kinase. *Mol Cell Biol* 1997; **17**: 1947–1958.
31. El Benna J, Dang PM, Gougerot-Pocidalo MA, Marie JC, Braut-Boucher F. p47phox, the phagocyte NADPH oxidase/NOX2 organizer: structure, phosphorylation and implication in diseases. *Exp Mol Med* 2009; **41**: 217–225.
32. Ushio-Fukai M. Compartmentalization of redox signaling through NADPH oxidase-derived ROS. *Antioxid Redox Signal* 2009; **11**: 1289–1299.
33. Naughton R, Quiney C, Turner SD, Cotter TG. Bcr-Abl-mediated redox regulation of the PI3K/AKT pathway. *Leukemia* 2009; **23**: 1432–1440.
34. O'Brien JJ, Spinelli SL, Tober J, Blumberg N, Francis CW, Taubman MB *et al.* 15-deoxy-delta12,14-PGJ2 enhances platelet production from megakaryocytes. *Blood* 2008; **112**: 4051–4060.
35. Hernandez-Hernandez A, Rodriguez MC, Lopez-Revuelta A, Sánchez-Gallego JI, Shnyrov V, Llanillo M *et al.* Alterations in erythrocyte membrane protein composition in advanced non-small cell lung cancer. *Blood Cells Mol Dis* 2006; **36**: 355–363.
36. Diaz-Hernandez JI, Almeida A, Delgado-Esteban M, Fernandez E, Bolanos JP. Knockdown of glutamate-cysteine ligase by small hairpin RNA reveals that both catalytic and modulatory subunits are essential for the survival of primary neurons. *J Biol Chem* 2005; **280**: 38992–39001.
37. Carcamo-Orive I, Tejados N, Delgado J, Gaztelumendi A, Otaegui D, Lang V *et al.* ERK2 protein regulates the proliferation of human mesenchymal stem cells without affecting their mobilization and differentiation potential. *Exp Cell Res* 2008; **314**: 1777–1788.

Supplementary Information accompanies the paper on Cell Death and Differentiation website (http://www.nature.com/cdd)