Abscisic acid (ABA) is a phyt hormone involved in pivotal physiological functions in higher plants. Recently, ABA has been proven to be also secreted and active in mammals, where it stimulates the activity of innate immune cells, mesenchymal and hematopoietic stem cells, and insulin-releasing pancreatic β cells through a signaling pathway involving the second messenger cyclic ADP-ribose (cADPR). In addition to behaving like an animal hormone, ABA also holds promise as a nutraceutical plant-derived compound in humans. Many biological functions of ABA in mammals are mediated by its binding to the LANCL-2 receptor protein. A putative binding of ABA to GRP78, a key regulator of endoplasmic reticulum stress, has also been proposed. Here we investigated the role of exogenous ABA in modulating thrombopoiesis, the process of platelet generation. Our results demonstrate that expression of both LANCL-2 and GRP78 is up-regulated during hematopoietic stem cell differentiation into mature megakaryocytes (Mks). Functional ABA receptors exist in mature Mks because ABA induces an intracellular Ca\(^{2+}\) increase ([Ca\(^{2+}\)]\(_i\)) through PKA activation and subsequent cADPR generation. In vitro exposure of human or murine hematopoietic progenitor cells to 10 \(\mu\)M ABA does not increase recombinant thrombopoietin (rTpo)-dependent Mk differentiation or platelet release. However, under conditions of cell stress induced by rTpo and serum deprivation, ABA stimulates, in a PKA- and cADPR-dependent fashion, the mitogen-activated kinase ERK 1/2, resulting in the modulation of lymphoma 2 (Bcl-2) family members, increased Mk survival, and higher rates of platelet production. In conclusion, we demonstrate that ABA is a prosurvival factor for Mks in a Tpo-independent manner.

Abscisic acid (ABA)\(^2\) is a hormone involved in many physiological and developmental processes throughout the life cycle of plants. In the early phase of the life of a plant, ABA regulates seed maturation and the maintenance of embryo dormancy (1, 2). Later, at the onset of ontogenesis, it mediates several adaptive responses toward environmental cues such as desiccation, cold, or salt stress and acts as a negative growth regulator. Recently, ABA has been shown to be also present and active in mammals, where it stimulates the functional activity of innate immune cells (3, 4), insulin biosynthesis, release in pancreatic β cells, and glucose uptake by adipocytes and myoblasts (5, 6). In the bone marrow (BM), high levels of ABA were detected in mesenchymal stem cells, which play an essential role in the BM microenvironment by providing hematopoietic progenitors with soluble factors essential to their proliferation and differentiation and to prevent detrimental lymphocyte activation (7). Consistently, micromolar amounts of ABA were demonstrated to expand uncommitted human hematopoietic progenitors (8).

Because of these potentially beneficial effects and its presence in fruits and vegetables, ABA is currently investigated as a possible nutraceutical compound (9). In this regard, recent evidence has been provided regarding the effects of low-dose ABA intake, in the form of fruit extracts, on the improvement of glucose tolerance and decrease of insulinemia in rats and humans (10).

ABA effects on mammalian cells are dependent on the G protein-coupled and peripheral membrane protein lanthionine synthetase C-like protein 2 (LANCL-2) (11–13). Binding of ABA to LANCL-2 triggers activation of a PKA-mediated signaling pathway involving the ADP-ribosyl cyclase-catalyzed conversion of NAD\(^+\) to the second messenger and potent Ca\(^{2+}\) mobilizer cyclic ADP-ribose (cADPR) (14–17). Recently, members of the heat shock protein 70 family of chaperones (including GRP78 and HSP70-2) have been identified as putative ABA binding proteins (18). The functional and mechanistic significance of an interaction between ABA and HSP-70 proteins in mammalian cells remains unknown (18). However, the increasingly recognized role of GRP78 as a key marker and mechanistic player of the unfolded protein response triggered by several endoplasmic reticulum (ER) stress conditions (19–22) cer-
The Role of Abscisic Acid in Thrombopoiesis

certainly demands further investigations of possible ABA-GRP78 interactions.

Megakaryocytes (Mks) differentiate from stem cells and are responsible for platelet release into the bloodstream (23). During differentiation, Mks become giant and polyploid cells under the effects of thrombopoietin (Tpo), the main thrombopoietic factor in the BM. Tpo acts by binding to a specific cell surface receptor, the cellular homolog of the myeloproliferative leukemia virus oncogene (Mpl), leading to receptor dimerization, activation of intracellular signal transduction pathways, and responses of target cells. Many of the effects of Tpo on cell survival and proliferation have been ascribed to the activation of the Jak/STAT and Ras/Raf/MAPK pathways (24). The use of recombinant Tpo (rTpo) has facilitated the development of in vitro Mk culture systems, improving the study of the mechanisms of platelet formation. However, its role in this process is still debated (25).

At the end of their maturation process, Mks extend long branching processes, designated proplatelets, into sinusoidal blood vessels where platelets are released (26, 27). Although platelet release shares several features with the apoptotic process, recent findings have demonstrated that platelet production proceeds independently of both the intrinsic and extrinsic apoptotic pathways (28–32).

Here we demonstrate that in vitro differentiated Mks express functional LANCL-2. Moreover, ABA promotes Mk survival in a Tpo-independent manner through ERK 1/2-dependent modulation of Bcl-2 family members implicated in the regulation of cell apoptosis.

Results

Expression of ABA-binding Proteins during Thrombopoiesis—After the cloning and characterization of Tpo, several in vitro culture systems have been developed to obtain highly enriched populations of Mks. Under our well characterized culture conditions, human Mks are differentiated with recombinant human Tpo (rhTpo), IL-11, and IL-6 in serum-free medium, whereas mouse Mks are differentiated in serum-containing medium in the presence of recombinant mouse Tpo (rmTpo) only (33, 34).

Specifically, in this study, enriched CD34+ hematopoietic stem cells were purified from cord blood and differentiated for 13 days in the presence of rhTpo, IL-11, and IL-6 to generate mature CD34+, CD41+, CD42b+ Mks (Fig. 1, A and B). Under these culture conditions, expression of the ABA main receptor LANCL-2 and of the ABA-binding protein GRP78 was measured in freshly isolated CD34+ cells and cells on days 7, 10, and 13 of Mk differentiation. Increased expression of GRP78 and LANCL-2 mRNAs was detected during Mk maturation, with a peak on day 10 of differentiation (p < 0.001 and 0.05 for the two transcripts, respectively, compared with time 0) and a subsequent, comparable fall on day 13 (Fig. 1C). The time-dependent pattern of GRP78 changes confirms the earlier results of Lopez et al. (35), who suggested that transient up-regulation of GRP78, peaking on day 11 of Mk maturation, marks the shift of the first step of differentiation to the stage of proplatelet formation. In addition, they demonstrated that these patterns of GRP78 expression indicate a parallel transient activation of ER stress, which seems to be strictly required for Mk maturation (35).

The increased synthesis of GRP78 and LANCL-2 was confirmed by Western blotting analysis in CD61 (β3 integrin)-positive Mks (Fig. 1D). As shown in Fig. 1E, Mks cultured in the presence of 10 μM ABA showed higher levels of LANCL-2 and GRP78 proteins at the end of culture (day 13). In addition, short (16-h) stimulation of mature Mks with ABA only was sufficient to induce a significant increase in LANCL-2 and GRP78 mRNAs (p < 0.05) (Fig. 1F) and a slight increase in protein expression (Fig. 1G). Overall, these results demonstrated that differentiated Mks express both ABA-binding proteins whose levels can be further and steadily up-regulated in an autocrine manner by the addition of ABA to the culture medium.

ABA Promotes a Cytosolic Ca2+ Increase in Mature Mks—We demonstrated previously that ABA evokes, in mammalian cells, a LANCL-2-initiated signaling cascade characterized by the two-step activation of PKA and the PKA substrate and the ADP-ribosyl cyclase enzyme CD38, with the sequential generation of the 2 s messengers cAMP and cADPR and resulting in a rise in intracellular Ca2+ concentration ([Ca2+]i) (3–5). To test the functionality of LANCL-2 in human Mks, cells on day 13 of culture were seeded in fresh medium only and stimulated with 10 μM ABA to analyze changes in the phosphorylation levels of PKA substrates. As shown in Fig. 2A, increased phosphorylation of PKA substrates was detected after 15, 30, and 60 min of ABA stimulation. The specificity of ABA effects on cell activation was further confirmed by the reduced phosphorylation of PKA substrates in Mks pretreated with a well known PKA inhibitor (H89) prior to ABA stimulation (Fig. 2A). Next, the effects of 10 μM ABA on cytosolic Ca2+ concentration were evaluated. Addition of ABA to mature human Mks induced a significant increase in [Ca2+]i, compared with controls (water; Fig. 2, B and C). The [Ca2+]i increase was almost completely inhibited by preincubation of the cells with the cADPR antagonist 8-Br-cADPR or the PKA inhibitor H89 (Fig. 2, D and E), confirming LANCL-2 functionality and involvement of cAMP and cADPR in eliciting the ABA-induced and Ca2+ -dependent effects on Mks (see below).

ABA Does Not Synergize with Cytokines in Sustaining Mk Differentiation and Platelet Release—To define possible effects of ABA on Mk differentiation, 10 μM ABA was added to the standard culture medium used to differentiate human and mouse Mks from their progenitors. As shown in Fig. 3, the addition of ABA did not enhance the percentage of Mks differentiating from both human and mouse hematopoietic progenitor cells (Fig. 3, A and B). The maturation profile of human Mks was similar in control and ABA-treated cultures, as comparable levels of low and high ploidy Mks were detected by flow cytometry at the end of culture (Fig. 3C). Consistently, when human mature Mks were seeded in fresh medium containing rhTpo for an additional 24 h in the presence or absence of ABA, no differences were observed in the percentage of proplatelet formation (Fig. 3, D and E) or in the rate of platelet release, as quantified with cell counting beads by flow cytometry (Fig. 3, F and G). Altogether, these data demonstrate that ABA does not increase/amplify Mk differentiation, maturation, and platelet production under standard culture conditions in vitro. However, the
finding that ABA significantly increases the [Ca²⁺] in mature Mks when seeded in fresh medium without Tpo and other differentiating factors (Fig. 2) prompted us to focus on the mechanisms of ABA signaling on fully differentiated cells in the absence of culture supplements (e.g., cytokines and serum).

ABA Increases Survival of Mature Mks in a Tpo-independent Manner by Up-regulating ERK 1/2 and the Expression of Anti-apoptotic Bcl-2 Family Members—Tpo deprivation in vitro has been demonstrated to reduce Mks viability and increase apoptosis (36). In plants, ABA regulates cell survival during stress conditions and induces the phosphorylation of several components of the MAPK cascade (37). To determine whether ABA can induce activation of MAPKs in human Mks independently of Tpo, we investigated the phosphorylation levels of the ERK 1/2 and p38 MAP kinases in Mks differentiated for 13 days in the presence or absence (Ctrl, water) of 10 μM ABA. ABA was added at the beginning of the culture and with every medium change on days 3, 7, and 10 of differentiation.

As shown in Fig. 4A, differentiated human Mks displayed rapid and sustained phosphorylation of ERK 1/2 in the presence of 10 μM ABA with respect to untreated Mks. These effects were dose-dependent, as only micromolar concentrations of ABA were able to induce ERK 1/2 phosphorylation (Fig. 4B). On the contrary, the phosphorylation level of p38 MAPK was not affected by ABA stimulation (Fig. 4C). To test whether ERK 1/2 activation was dependent on the upstream PKA/CD38 pathway, the phosphorylation levels of ERK 1/2 and p38 MAPK were evaluated in Mks treated with specific inhibitors. Consistently with results on [Ca²⁺] increase (Fig. 2, D and E), phosphorylation of ERK 1/2 was significantly reduced by antagonizing both PKA activity (with H89) and cADPR function (with 8-Br-cADPR) in Mks stimulated with ABA for 30 min (Fig. 4D).

Together, these findings raise the possibility that the PKA/CD38 pathway targets the MEK/ERK 1/2 pathway to sustain ABA-induced and cADPR-mediated modulation of gene transcription. In mammals, ERK 1/2 is implicated in the promotion of cell survival through the regulation of antiapoptotic protein expression (38), and, recently, ABA has been demonstrated to induce apoptosis in glioblastoma cell lines (39). Thus, we studied the ability of ABA to induce the transcription of ERK 1/2 target genes to address a potential prosurvival role of ABA in stressed Mks. Therefore, differentiated human Mks were deprived of rhTpo and cultured in fresh medium in the presence or absence of 10 μM ABA for 24 h. Members of the Bcl-2

FIGURE 1. Expression of ABA-binding proteins during human thrombopoiesis. A, flow cytometry analysis of CD34 and CD41 cell marker expression in freshly isolated CD34⁺ hematopoietic stem cells (HSCs) and after 13 days of differentiation toward the Mk lineage. B, percentages of CD34⁺ or CD41⁺ cells in freshly isolated CD34⁺ HSCs and after 13 days of differentiation toward the Mk lineage. Results from three independent experiments are shown. C, RT-PCR of LANCL-2 and GRP78 expression in CD34⁺ HSCs and Mks on days 3, 7, 10, and 13 of differentiation. At least three independent experiments were performed. Data are presented as mean ± S.D. *p < 0.05; ***, p < 0.001. D, representative Western blot of LANCL-2 and GRP78 expression on days 7, 10, and 13 of Mk differentiation. Calreticulin was revealed to ensure equal protein levels, whereas β3 integrin was revealed to monitor Mk differentiation. OD, optical density. E, analysis of LANCL-2 and GRP78 protein levels in Mks differentiated for 13 days in the presence or absence (Ctrl, water) of 10 μM ABA. ABA was added at the beginning of the culture and with every medium change on days 3, 7, and 10 of differentiation. F, RT-PCR analysis of LANCL-2 and GRP78 mRNA levels in mature Mks stimulated or not (Ctrl, water) for 16 h with 10 μM ABA. Three independent experiments were performed. Data are presented as mean ± S.D. *p < 0.05. G, representative Western blot analysis of LANCL-2 and GRP78 expression in mature human Mks stimulated or not (Ctrl, water) for 16 h with 10 μM ABA.
The Role of Abscisic Acid in Thrombopoiesis

family with antiapoptotic activity (Bcl-2 and Bcl-XL), but not with proapoptotic activity (Bax), were significantly up-regulated at both the mRNA and protein levels after 24 h of ABA treatment (Fig. 4, F and H). Further, inhibition of PKA and cADPR function by H89 (10 μM) and 8-Br-cADPR (50 μM), respectively, significantly reduced the ABA-dependent effects on expression of the antiapoptotic proteins Bcl-2 and Bcl-XL in human Mks (Fig. 4, J and J, p < 0.001). Overall, these data demonstrated that, independent of Tpo, ABA may function as a signal to promote Mk survival through ERK 1/2 activation and modulation of the pro/antiapoptotic protein ratio.

The cADPR-dependent [Ca^{2+}]_i Increase in ABA-treated Mks Is Mediated by Ryanodine Receptors and Not by TRPM-2 Activation—cADPR has been recognized as a universal Ca^{2+} mobilizer by activating ryanodine receptors (RyRs) in many types of cells (16, 17). In addition, cADPR has been reported to mediate Ca^{2+} entry by activating transient receptor potential cation channel melastatin 2 (TRPM-2) (16, 17, 40). To investigate whether RyRs or TRPM-2 channels are involved in the cADPR-induced increase in [Ca^{2+}]_i, we first evaluated the expression of these receptors in human Mks by RT-PCR. Our results demonstrated that RyR1 and RyR3 were expressed in differentiated human Mks on day 13 of culture, whereas RyR2 was not detected under the same experimental conditions (Fig. 5A). TRPM-2 was also expressed by terminally differentiated human Mks (Fig. 5A). Expression of RyRs was further confirmed by RT-PCR in mouse BM-derived Mks (data not shown). However, RyRs were hardly detectable by Western blotting analysis in human and mouse MK extracts, probably because of the low abundance with which the RyRs are expressed within these cells (data not shown).

These data prompted us to investigate, at a functional level, whether the ABA effects on mature Mks were dependent on Ca^{2+} mobilization from intracellular stores or on extracellular Ca^{2+} entry. For this purpose, we first measured the [Ca^{2+}]_i in Mks loaded with the Ca^{2+}-sensitive fluorochrome Fura-2 in the absence of extracellular Ca^{2+} (0 Ca^{2+}) prior to stimulation with 10 μM ABA (Fig. 5B). Interestingly, when ABA was applied in 0 Ca^{2+}, the initial increase in [Ca^{2+}]_i, still occurred (peak intensity, see the legends for Figs. 2 and 5 for details), whereas the following plateau phase (peak duration, see the legend for Fig. 5) was significantly decreased (Fig. 5, B, D, and E; p < 0.001). Thus, intracellular Ca^{2+} release plays a major role in eliciting the Ca^{2+} peak induced by ABA, whereas extracellular Ca^{2+} influx might be involved in sustaining a late phase of Ca^{2+} signaling.

In the second approach, we performed fluorescence measurements of Ca^{2+} in mature Mks pretreated with the RyRs inhibitor tetracaine or with the specific TRPM-2 inhibitor econazole prior to ABA stimulation (Fig. 5C). The results confirmed a prominent role of RyRs in mediating cADPR-dependent effects in Mks stimulated with ABA, as tetracaine completely abrogated both the peak intensity and duration of the intracellular Ca^{2+} increase in treated Mks (Fig. 5, D and E; p < 0.001). On the contrary, inhibition of TRPM-2 by econazole in ABA-treated cells did not affect the early peak of calcium increase but significantly decreased the plateau phase to a value comparable with that observed in Mks stimulated with ABA in 0 Ca^{2+} (Fig. 5, D and E, p < 0.001). Overall, these data support the view that Ca^{2+} increase after ABA stimulation is mediated by an initial Ca^{2+} mobilization from ER stores through RyRs activation, whereas TRPM-2 sustains Ca^{2+} entry from the extracellular...
On the contrary, inhibition of Ca\textsuperscript{2+} mobilization of Ca\textsuperscript{2+} and gene transcription in human Mks are dependent on the observed effects of ABA on the modulation of ERK 1/2 activity (Fig. 5, not affect the phosphorylation level of ERK 1/2 after ABA stimulation were evaluated in standard conditions (Ctrl) or with addition of 10 μM ABA. ABA was added at the beginning of the culture and with every medium change on days 3, 7, and 10 of differentiation. N, nuclei. D, representative phase-contrast images of human Mk-forming proplatelets seeded in fresh medium for 16 h in the presence of 10 ng/ml rhTpo alone or rhTpo plus exogenous 10 μM ABA, and proplatelet formation was quantified (mean ± S.D., n = 3 separate experiments). P, on day 13 of maturation, human Mks were seeded in fresh medium for 16 h in the presence of rhTpo alone or rhTpo plus 10 μM ABA. Samples were mixed with counting beads to quantify the absolute number of released platelets (PLT) by flow cytometry. Platelets were identified as CD41\textsuperscript{+} events in the FL1 green channel with the same physical parameters of peripheral blood platelets from a control subject, whereas beads were visualized in the FL2 detector (red channel). SSC, side scatter; FSC, forward scatter; B, beads; P, platelets. G, absolute numbers of CD41\textsuperscript{+} released platelets analyzed by flow cytometry. On day 13 of maturation, 2 × 10\textsuperscript{5} human Mks were seeded in fresh medium for 16 h in the presence of rhTpo alone or rhTpo plus 10 μM ABA. Results are presented as mean ± S.D. n = 3 separate experiments.

ABA Increases Mk Survival under Stress Conditions and Sustains Prolonged Platelet Release in Vitro—To determine whether ABA has a measurable effect on Mk survival, we explored the ability of ABA to increase the survival of differentiated Mks under conditions of cell stress. For this purpose, human Mks at the end of culture were reseeded under Tpo-deprived conditions, whereas mouse Mks differentiated from fetal liver progenitor cells were purified with a BSA gradient and cultured under Tpo- and serum-deprived conditions. Under both experimental conditions, Mk properties were monitored over time for 2 additional days in the absence or presence of 10 μM ABA. Cell viability was evaluated by staining the cells with a specific Mk marker (CD41 or CD45) and measuring the exclusion of the DNA dye 7-amino actinomycin D (7-AAD) by flow cytometry. As shown in Fig. 6, A and B, ABA increased the rate of Mk survival, with a peak after 48 h in human Mks and after 24 h in mouse Mks. Moreover, the increased survival rate under conditions of cell stress in ABA-treated Mks was paralleled by a significant increase at 48 h in the percentage of mouse Mks forming proplatelets with respect to the untreated control (Fig. 6C). Consistently, the absolute number of released plate-

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**FIGURE 3.** ABA does not modulate Tpo-dependent differentiation, maturation, and platelet release in human and mouse Mks. A, Flow cytometry analysis of percentages of CD41\textsuperscript{+} Mks differentiated from human cord blood-derived CD34\textsuperscript{+} cells in standard cultures (Ctrl) or with addition of 10 μM ABA for 13 days. ns, not significant. B, flow cytometry analysis of percentages of CD41\textsuperscript{+}CD45\textsuperscript{+} cells in standard cultures (Ctrl) or with addition of 10 μM ABA. ABA was added at the beginning of the culture and with every medium change on days 3, 7, and 10 of differentiation. C, analysis of cell ploidy in human Mks differentiated in standard cultures (Ctrl) or with addition of 10 μM ABA. ABA was added at the beginning of the culture and with every medium change on days 3, 7, and 10 of differentiation.
The Role of Abscisic Acid in Thrombopoiesis

**FIGURE 4. ABA induces ERK phosphorylation and modulation of anti-apoptotic Bcl-2 family proteins in a Tpo-independent manner.** A, representative time course analysis of ERK 1/2 phosphorylation levels in Tpo-deprived human Mks in the presence (ABA) or absence of 10 μM ABA. Vinculin was revealed to ensure equal protein loading. Bottom panel, a graph quantifying the ratio of phosphorylated ERK 1/2 and total ERK 1/2. Results from three independent experiments are presented. ***, p < 0.001. B, dose-dependent analysis of ERK 1/2 phosphorylation after 30 min in control Mks or Mks stimulated with ABA at 1, 10, and 100 ng/ml or 2 and 10 μg/ml concentrations. Bottom panel, a graph quantifying the ratio of phosphorylated ERK 1/2 and total ERK 1/2. Results from three independent experiments are presented. ***, p < 0.001. C, representative time course analysis of p38 phosphorylation levels in Tpo-deprived human Mks stimulated with 10 μM ABA or not. Vinculin was revealed to ensure equal protein loading. Bottom panel, a graph quantifying the ratio of phosphorylated p38 and total p38. Results from three independent experiments are presented. ***, p < 0.001. D, representative Western blotting analysis of ERK 1/2 phosphorylation in control Tpo-deprived human Mks (water) and Mks stimulated with 10 μM ABA or not. Vinculin was revealed to ensure equal protein loading. Bottom panel, a graph quantifying the ratio of phosphorylated ERK 1/2 and total ERK 1/2 of at least three independent experiments. *, p < 0.05; **, p < 0.01; ***, p < 0.001. E, representative Western blotting analysis of p38 phosphorylation in control Tpo-deprived human Mks (water), Mks stimulated with 10 μM ABA for 30 min, and Mks pretreated with 8-Br-cADPR (50 μM) or H89 (10 μM) prior to ABA stimulation. Bottom panel, a graph quantifying the ratio of phosphorylated p38 and total p38 of at least three independent experiments. *, p < 0.05; **, p < 0.01. ***, p < 0.001. F, RT-PCR analysis of Bcl-2, Bcl-XL, and Bax mRNA levels in Tpo-deprived human Mks treated (ABA) or not (Ctrl, water) with 10 μM ABA for 24 h (mean ± S.D., n = 3 separate experiments). *, p < 0.05; **, p < 0.01. G,cord blood-derived human Mks were cultured overnight in the absence of rhTPO and stimulated with exogenous 10 μM ABA or not (Ctrl) for 24 h. The protein levels of Bcl-2, Bcl-XL, and Bax were analyzed by Western blotting. A representative experiment is shown. H, quantification of Bcl-2, Bcl-XL, and Bax by relative densitometric analysis of the protein/vinculin ratio in a Western blot of control or control ABA-treated Mks after 24 h of culture in the absence of rhTPO. Results are expressed as mean ± SD of three independent experiments. ***, p < 0.001. J, Western blotting analysis of Bcl-2, Bcl-XL, and Bax in cord blood-derived human Mks cultured overnight in the absence of Tpo and presence of 10 μM ABA or absence (Ctrl) for 24 h or treated with 8-Br-cADPR (50 μM) or H89 (10 μM) prior to ABA stimulation. J, quantification of Bcl-2, Bcl-XL, and Bax protein levels in Mks stimulated with ABA in the absence of Tpo or pretreated with 8-Br-cADPR (50 μM) or H89 (10 μM) prior to ABA stimulation. At least three independent experiments were performed. ***, p < 0.001.

lets was increased in mouse Mk cultures treated with ABA with respect to the untreated control after 48 h (Fig. 6, D and E, p < 0.05). Finally, to elucidate whether the prosurvival effects of ABA on Mks was dependent on activation of the mitogen-activated kinase ERK 1/2, human mature Mks were pretreated with a specific ERK 1/2 inhibitor, PD98059 (10 μM), prior to treatment with 10 μM ABA for 24 h. Increased expression of antiapoptotic proteins (Bcl-2 and Bcl-XL) induced by ABA was significantly abrogated by ERK 1/2 inhibition (Fig. 6, F and G, p < 0.001). Further, as shown in Fig. 6, H and I, ERK 1/2 inhibition significantly reduced the beneficial effects of ABA in terms of cell survival, as measured by exclusion of the dye 7-AAD by flow cytometry. These results clearly demonstrate that ABA can act as an Mk prosurvival agonist in response to cytokine and serum deprivation through an ERK 1/2-dependent mechanism.

**Discussion**

ABA is a phytohormone involved in the control of several physiological and developmental processes in higher plants (1–2). We have recently demonstrated that ABA is produced and released by several human cell types, which also respond to this hormone with functional activities triggered by cytosolic Ca2+ increases. The pathways of ABA biosynthesis have been widely explored and elucidated in plants, but less is known in animals. Concerning the mechanisms of ABA release in mammalian cells, it has been reported recently that ABA transport is mediated by members of the anion exchanger (AE) family of anion exchangers and is bidirectional (41, 42). This feature accounts for ABA uptake and release from mammalian and human cells, and, more generally, for the mechanisms of ABA-related autocrine and paracrine functions in the animal kingdom (43). Established sources of plasmatic ABA, in addition to dietary intake of fruits and vegetables (10), are pancreatic β cells, adipocytes, and, to a lesser extent, granulocytes, monocytes, and mesenchymal stem cells (43).

Despite the completely different ABA receptors in plants and humans, the remarkable conservation of cADPR-Ca2+ signaling pathways from vegetal (44) to animal kingdoms demon-
strates that ABA can be considered a universal signaling molecule (3–8). In in vivo models, administration of ABA was demonstrated to have beneficial effects on several conditions and diseases, including obesity-related inflammation, diabetes, atherosclerosis, and inflammatory bowel disease (10, 45–48). Here we demonstrate, for the first time, that exogenous ABA can act as a prosurvival factor for Mks, leading to increased platelet generation and release in a Tpo-independent manner. Our results demonstrate that the expression of the main ABA receptor in mammalian cells, LANCL-2, increases during Mk development and that addition of exogenous ABA to mature Mks elicits a signaling pathway involving PKA/cAMP and cADPR-mediated cytosolic Ca²⁺ increase, as described previously in other human cell types (3–5). Next, we show that, different from previous studies in rats (49), human and mouse Mks express functional RyRs that are responsible for the cytosolic Ca²⁺ increase mediated by ABA.

The failure to detect RyRs proteins by Western blotting, despite identification of the two corresponding transcripts by RT-PCR, can therefore be ascribed to their low abundance in Mks. In control human Mks stimulated with 10 μM ABA, stimulated with 10 μM ABA, or pretreated with 100 μM tetracaine or 10 μM econazole prior to ABA stimulation. Peak duration was calculated as time elapsed between the achievement of maximum fluorescence intensity and return to the ground fluorescence intensity. Results are presented as mean ± S.D. of four independent experiments (total number of cells analyzed, n = 310). ***, p < 0.001. L, representative Western blotting analysis of Bcl-2, Bcl-XL, and Bax in cord blood-derived human Mks cultured with 10 μM ABA or water (control) for 24 h or treated with tetracaine (100 μM) prior to ABA stimulation. M, quantification of Bcl-2, Bcl-XL, and Bax protein levels in human Mks stimulated with water (Ctrl) or 10 μM ABA or pretreated with tetracaine (10 μM). Three independent experiments were performed.
Ca\(^{2+}\) signaling. This observation is coherent with the notion that intracellular Ca\(^{2+}\) acts synergistically with cADPR (or ADPR) to gate TRP-2 channels (50). This feature strongly suggests that ABA is unlikely to gate TRPM-2 without the prior stimulation of RyRs-dependent Ca\(^{2+}\) mobilization. Alternatively, TRPM-2-mediated Ca\(^{2+}\) entry could be locally amplified by RyRs through the Ca\(^{2+}\)-induced Ca\(^{2+}\) release process, as observed for TRP vanilloid 4 channels (51). In this case, upon RyR blockade with tetracaine, TRPM-2-dependent Ca\(^{2+}\) signals could remain confined beneath the plasma membrane and fall below the resolution of our Ca\(^{2+}\) imaging system.

Further, we show that, in the unique and complex biological process of Mk development, ABA does not synergize with Tpo in supporting Mk differentiation, maturation, or platelet release. However, in differentiated Mks and under conditions of cellular stress, stimulation with ABA induces, through the cAMP/cADPR signaling cascade, the peculiar activation of the mitogen activated kinase ERK 1/2, resulting in the up-regulation of antiapoptotic Bcl-2 family members, thus increasing Mk survival and increasing platelet release in vitro in the absence of recombinant Tpo and serum.

The involvement of members of the Bcl-2 protein family in apoptotic mechanisms triggered by micromolar ABA is not unprecedented. An earlier study has demonstrated a significant up-regulation of Bcl-2 in human uncommitted hemopoietic progenitors (CD34 cells) following incubation with 2 μM ABA for 24 h (8). More recently, ABA has been reported to induce apoptosis in glioblastoma cells via up-regulation of Bax and concomitant down-regulation of Bcl-2 compared with ABA-untreated cells (39). These ABA-induced effects, opposite to the pro-survival outcome observed in our study with stressed Mks, are mediated by other players of apoptosis/differentiation and especially by the involvement of the retinoic acid signaling pathway (39). Conversely, the role of ERK 1/2 in ABA-responsive glioblastoma cells was not investigated. Finally, a recent study demonstrated that, in human mesenchymal stem cells, cADPR stimulates cell proliferation by inducing repetitive Ca\(^{2+}\) oscillations that, in turn, lead to phosphorylation of ERK 1/2 (52). Therefore, it appears that, in different types of human cells, cADPR, either autocorinally generated (52) or intracellularly produced as a second messenger of the ABA signaling cascade (this study), expands the stem cell precursors through...
multiple, still ill-defined mechanisms downstream of ERK 1/2 activation. Indeed, the ERK 1/2 pathway is known to be associated with increased cell proliferation (52) but also with decreased apoptosis (53, 54), depending on different cell types and context-specific conditions (55).

Tpo, also known as c-Mpl ligand, is the primary physiological growth factor for the Mk lineage that also plays a central role in the survival and proliferation of hematopoietic stem cells (56, 57). However, although Tpo is one key driver of Mk differentiation, mice lacking either c-Mpl or Tpo are able to successfully produce platelets, indicating a role for other regulators in the end stage of Mk maturation (58, 59). ABA did not synergize with Tpo to sustain or increase Mk differentiation, but its functional consequences on Mk signaling and function become evident only after deprivation of Tpo and serum from the media of human and mouse Mks cultures. These effects might be explained by the ability of ABA to activate in Mks the same biochemical pathways normally regulated by Tpo, such as a rapid and sustained ERK 1/2 phosphorylation (60) as well as Ca\(^{2+}\) signaling (61). Therefore, as in plants, the beneficial effects of ABA on Mks and platelet production are prominent when cells experience a transient stress condition, such as the withdrawal of their main physiological regulator.

Data on the signals that drive terminal Mk maturation are still insufficient to elucidate the exact mechanisms of platelet production. It is known that Ca\(^{2+}\) fluxes play a crucial role in the regulation of mature Mk functions and platelet formation (62), whereas the balance between cell survival, apoptosis, and platelet biogenesis in Mks is still debated and far from being completely deciphered (28–32). An example of such complexity is a different time schedule between Mk survival and pro-platelet formation in ABA-treated human and murine Mks, respectively (Fig. 6, platelet formation in ABA-treated human and murine Mks, FEBRUARY 24, 2017 • VOLUME 292 • NUMBER 8

A significant source of BM-derived ABA is represented by mesenchymal stem cells and other hematopoietic lineages as well as by granulocytes and macrophages (3, 7), which are closely associated with Mks in the BM, thereby ensuring overall BM homeostasis (64).

Interestingly, ABA might be autocrinally released by Mks, as demonstrated by the detection of ABA in a range of 0.5–1.5 pmol/10\(^6\) cells in human Mk extracts on day 13 of culture (data not shown). This suggest that in vivo Mk-ABA interactions occur in vivo. With respect to this, we have demonstrated previously that a significant source of BM-derived ABA is represented by mesenchymal stem cells and other hematopoietic lineages as well as by granulocytes and macrophages (3, 7), which are closely associated with Mks in the BM, thereby ensuring overall BM homeostasis (64).

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The use of micromolar concentrations of ABA in this study, remarkably higher than the nanomolar basal levels of ABA detectable in human plasma (10), does not exclude that functionally active concentrations may be reached in vivo under specific conditions and at selected sites of the organism (e.g. in the BM hematopoietic niche). Moreover, our recent study on administration in rats and humans of low-dose dietary ABA (0.5–1.0 \(\mu\)g/kg of body weight) demonstrates that nanomolar

The Role of Abscisic Acid in Thrombopoiesis

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plasma concentrations were functionally effective to the extent of improving glucose tolerance and decreasing insulinemia, whereas much higher doses of ingested ABA (50 mg/kg of body weight) resulted in enhanced insulin release (10). This discrepancy provides evidence that in vitro experiments may not necessarily enable the prediction of the in vivo relevance of mechanisms of ABA effects in specific target cells, including Mk s. In this regard, there is a plethora of in vivo insults, including chemotherapy agents, autoantibodies, and viruses, that cause Mk stress, resulting in thrombocytopenia, reduced Mk survival, or increased apoptotic death (65–67). Conditions of reduced Tpo production have also been reported during liver diseases in which thrombocytopenia is caused not only by peripheral blood platelet destruction but also by decreased platelet production because of a reduction of mRNA for Tpo in the liver (65). On these grounds, it would be interesting to analyze the effects of ABA as a nutritional intervention during these circumstances of thrombopoietic stress (9, 10).

Finally, the positive effect of ABA on thrombopoiesis may be exploitable to improve the production of platelets in appropriate bioreactors of industrial interest, such as the recently obtained silk protein-based systems (68). Indeed, the silk model is exploitable to improve the production of platelets in appropriate bioreactors of industrial interest, such as the recently obtained silk protein-based systems (68). Indeed, the silk model is exploitable to improve the production of platelets in appropriate bioreactors of industrial interest, such as the recently obtained silk protein-based systems (68). Indeed, the silk model is exploitable to improve the production of platelets in appropriate bioreactors of industrial interest, such as the recently obtained silk protein-based systems (68). Indeed, the silk model is exploitable to improve the production of platelets in appropriate bioreactors of industrial interest, such as the recently obtained silk protein-based systems (68). 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5′-TCTCAAAGGTGACTTCAATC-3′, and TRPM2 forward 5′-TCGGACCAACACAGCTGTA-3′ and reverse 5′-CGTCATTCTGGTCTGGGAAGTG-3′. All were from Sigma-Aldrich. Predesigned primers for RyR1 (catalog no. HS00166991_m1), RyR2 (catalog no. HS00181461_m1), and RyR3 (catalog no. HS00168821_m1) were from Applied Biosystems (Thermo Fisher Scientific). CFX Manager® software 3.0 was used for normalization of the samples (Bio-Rad), β-2 microglobulin and GAPDH gene expression was used for the comparative concentration analysis.

Flow Cytometry—Cell viability was assessed by incubating Mk s with 5 μl of 7-AAD viability staining solution (BioLegend, Milan, Italy). Analysis of human Mks differentiation was performed on day 13 of culture. Mk s were identified as the percentage of CD41+ events in the fractions of CD45+ cells. For murine BM cultures, Mk s on day 4 of culture were analyzed using allophycocyanin-conjugated anti-mouse CD45 (clone 30F11, Miltenyi Biotech) and FITC-conjugated anti-mouse CD41 (clone M7/4, BioLegend) antibodies. Mk output was calculated as the percentage of side scatterhigh/forward scatterld/CD45+ /CD41+ cells and normalized to the total number of CD45+ cells. For Mk ploidy, cells were fixed overnight in ice-cold 70% ethanol at −20 °C. Samples were incubated in PBS with 100 μg/ml of RNase and propidium iodide solution and stained with FITC-conjugated anti-human CD41 antibody (clone H18, eBioscience, Milan, Italy). All samples were acquired with a Beckman Coulter Navios flow cytometer. Non-stained samples and isotype control antibodies were used to set the correct analytical gating. Offline data analysis was performed using the Beckman Coulter Navios software package.

Released Platelet Quantification—Platelets released in culture were quantified as described previously (69). Platelets produced in vitro were analyzed using the same forward and side scatter pattern as human and mouse peripheral blood platelets. Platelets were identified as CD41+, and their number was calculated using a TruCount bead standard by flow cytometry.

Measurement of Cytosolic Ca2+ Concentration—12-mm glass coverslips were coated overnight at 4 °C with 100 g/ml of fibronectin. On day 14 of culture, 1 × 10⁶ human Mks were harvested and allowed to adhere at 37 °C and 5% CO₂ for 16 h. Then Mk s were loaded with 4 μM Fura-2/AM (Molecular Probes Europe BV, Leiden, The Netherlands) in physiological salt solution (150 mM NaCl, 6 mM KCl, 1.5 mM CaCl₂, 1 mM MgCl₂, 10 mM glucose, and 10 mM HEPES (pH 7.4)) for an additional 30 min at 37 °C and 5% CO₂. After being washed in physiological salt solution, the coverslip was fixed to the bottom of a Petri dish, and the cells were observed using an upright epifluorescence AxioLab microscope (Carl Zeiss, Arse, Italy) equipped with a Zeiss X63 Achroplan objective (water immersion, 2.0 mm working distance, 0.9 numerical aperture). Mks were stimulated or not (control) with 10 μM ABA and excited alternately at 340 and 380 nm, and the emitted light was detected at 510 nm. A first neutral density filter (1 or 0.3 optical density) reduced the overall intensity of the excitation light, and a second neutral density filter (0.3 optical density) was coupled to the 380-nm filter to approach the intensity of the 340-nm light. The excitation filters were mounted on a filter wheel (Lambda 10, Sutter Instruments, Novato, CA). Custom software, working in the LINUX environment, was used to drive the camera (Extended ISIS camera, Photonic Science, Robertsbridge, UK) and the filter wheel to measure and plot the fluorescence from rectangular regions of interest, each enclosing every Mk present within the analyzed field. In some experiments, Mks were incubated with PKA (10 μM, 30 min at 37 °C), cADPR (50 μM, overnight at 37 °C), econazole (10 μM, 1 h at 37 °C) and tetracaine (100 μM, 1 h at 37 °C) inhibitors prior to ABA stimulation. Analysis of Ca²⁺ signals were performed according to methods published previously (62).

Statistics—For all experiments, values are expressed as mean ± S.D. Student’s t test was performed for paired observations. ANOVA followed by post hoc Bonferroni t test, was performed for grouped observations. p < 0.001, p < 0.01, or p < 0.05 were considered statistically significant. All experiments were independently replicated at least three times unless otherwise specified.

Author Contributions—A. M., C. F., C. A. D. B., and P. M. S. conducted the experiments and analyzed the results. F. M. analyzed the results. C. B., E. Z., A. D. F., and A. B. conceived the idea for the project. A. M. and A. B. wrote the paper.

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