Tight control of MEK-ERK activation is essential in regulating proliferation, survival, and cytokine production of CD34$^+$-derived neutrophil progenitors

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A plethora of extracellular stimuli regulate growth, survival, and differentiation responses through activation of the MEK-ERK MAPK signaling module. Using CD34$^+$ hematopoietic progenitor cells, we describe a novel role for the MEK-ERK signaling module in the regulation of proliferation, survival, and cytokine production during neutrophil differentiation. Addition of the specific MEK1/2 inhibitor U0126 resulted in decreased proliferation of neutrophil progenitors. Conversely, transient activation of a conditionally active MEK1 mutant resulted in the expansion of progenitor cells, which thereafter differentiated normally into mature neutrophils. In contrast, chronic MEK1 activation was found to induce cell death of CD34$^+$ neutrophil progenitors. Microarray analysis of CD34$^+$ progenitor cells showed that activation of MEK1 resulted in changes in expression of a variety of cell-cycle modulating genes. Furthermore, conditional activation of MEK1 resulted in a dramatic increase in the expression of mRNA transcripts encoding a large number of hematopoietic cytokines, chemokines, and growth factors. These findings identify a novel role for MEK-ERK signaling in regulating the balance between proliferation and apoptosis during neutrophil differentiation, and they suggest the need for tight control of MEK-ERK activation to prevent the development of bone marrow failure. (Blood. 2009; 114:3402-3412)

Introduction

Cytokines play an important role in the maintenance of hematopoietic homeostasis by regulating hematopoietic cell growth, survival, and differentiation through the activation of a number of intracellular signaling pathways that ultimately results in the activation of specific transcription factors and subsequent modulation of target gene expression.[1] The mitogen-activated protein kinase (MAPK) cascade is one of the key signaling pathways that couples the signals from cell-surface cytokine receptors to trigger downstream pathways.[2,3] Three major groups of MAPKs have been characterized in mammals, including extracellular signal-regulated protein kinases (ERKs), c-Jun NH_2-terminal kinases, and p38MAPKs.[4,5] Activation of MAPK is regulated by an evolutionary conserved kinase cascade and MAPKKKs are serine/threonine kinases capable of phosphorylating and activating MAPKKs, which in turn stimulate MAPK activity through dual phosphorylation on threonine and tyrosine residues within a tripeptide motif (Thr-X-Tyr). On activation, MAPK itself can phosphorylate specific target substrates on serine or threonine residues.[6,7]

The ERK MAPK is the most extensively studied MAPK family member that is best known for its role in the regulation of proliferation and survival of a plethora of cell types.[8-10] To date, 2 ERK kinases have been described, p44 ERK1 and p42 ERK2, that are 83% homologue.[11] They are ubiquitously expressed and are directly activated by the MAPKKs MEK1 and MEK2, whose only known substrates are ERK1 and ERK2.[4] Activation of ERKs occurs as a result of cytokine or growth factor stimulation, but they are also activated in response to hydrogen peroxide, UV light, and ionizing radiation [12-15] On activation, ERK1 and ERK2 translocate to the nucleus, where they phosphorylate and regulate a variety of nuclear targets, such as STAT3, Elk-1, c-fos, c-myc, and Ets transcription factors. Although most ERK substrates are nuclear proteins, others are found in the cytoplasm and organelles. The primary cytoplasmic target of the ERK kinases is p90 ribosomal S6 kinase, but a wide array of other targets are known to exist, including various membrane proteins, such as CD120a, Syk, and calnexin.[3,16,17]

Analysis of ERK MAPK–deficient mice has shown that ERK MAPK signaling plays an important role in the regulation of multiple cellular processes. ERK1$^{-/-}$ mice are viable, fertile, and of normal size, indicating that ERK2 may compensate for its loss.[18] However, although ERK1 apparently is dispensable during embryonic stages, ERK1$^{-/-}$ mice do exhibit impaired thymocyte development. In contrast, deletion of ERK2 was found to be embryonic lethal because of defects in trophoblast formation, mesoderm differentiation, and placental function, showing distinct biologic functions for ERK1 and ERK2.[19-21]

Evidence has accumulated showing that ERK signaling plays a fundamental role in regulating cellular proliferation at the G_1/S transition.[22] ERK1/2 activation is required for cyclin D1 expression, and sustained activity of ERK is also required for the down-regulation of many antiproliferative genes throughout the G_1 phase of the cell cycle.[23] In addition, the ERK MAPK pathway has profound effects on the regulation of apoptosis by

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the posttranslational phosphorylation of apoptotic regulatory molecules, including Bad, Bim, Mcl-1, and caspase 9.[8]

Although the ability of the ERK MAPK signaling module to promote cellular proliferation and survival has been well established, emerging evidence now suggests that it is also important for the differentiation of a variety of cell types. ERK MAPK has been shown to play a key role in the development of neuronal cells,[24] myoblasts,[25] and cells in the visual cortex.[26] In the hematopoietic system, ERK MAPK signaling has been shown to be essential for transition of double-negative thymocytes to double-positive stages,[27] as well as for megakaryocyte and erythrocyte differentiation.[28,29] In addition, a recent study indicated that ERK signaling may also play a role in lineage conversion of murine common lymphoid progenitors toward the myeloid lineage.[30]

Because the ERK MAPK signaling pathway plays an important role in the regulation of various cellular processes, it could be hypothesized that aberrant activation of this pathway may also cause hematopoietic disease in humans. The ERK MAPK pathway has been reported to be activated in more than 50% of acute myeloid leukemias and acute lymphocytic leukemias and is also frequently activated in other cancer types.[31,32]

In this study, we have investigated the role of the MEK-ERK MAPK signaling pathway in CD34+ hematopoietic progenitor cell function, focusing on neutrophil development, using a human ex vivo differentiation system. MEK-ERK activity was found to be an important regulator of both hematopoietic progenitor expansion and survival. Subsequent microarray analysis of CD34+ cells has shown that activation of MEK1 resulted in changes in expression of a variety of cytokine, chemokine, and cell-cycle genes, clarifying these observations. These findings identify a novel role for MEK-ERK signaling in regulating the balance between proliferation and apoptosis during neutrophil development, and they suggest the need for tight control of ERK activation because aberrant activation of this pathway could lead to the development of bone marrow failure.

Methods

Isolation and culture of human CD34+ cells

Mononuclear cells were isolated from umbilical cord blood by density centrifugation over a Ficoll-Paque solution (density 1.077 g/mL). Immunomagnetic cell separation (Miltenyi Biotec) was used to isolate CD34+ cells. CD34+ cells were cultured in Iscove modified Dulbecco medium (IMDM; Gibco) supplemented with 9% fetal calf serum (FCS; Hyclone), 50 μM β-mercaptoethanol, 10 U/mL penicillin, 10 μg/mL streptomycin, and 2 mM glutamine. Cells were differentiated toward neutrophils on the addition of stem cell factor (SCF; 50 ng/mL), FLT-3 ligand (50 ng/mL), granulocyte-macrophage colony-stimulating factor (GM-CSF; 0.1 nmol/L), interleukin-3 (IL-3; 0.1 nmol/L), and G-CSF (30 ng/mL). After 6 days of culture, only G-CSF was added to the cells. Erythrocytes were generated as described by Giarratana et al.[33] Briefly, CD34+ cells were cultured in IMDM supplemented with 1% BSA, 120 g/mL iron-saturated human transferrin, 900 ng/mL ferrous sulfate, 90 ng/mL ferric nitrate, and 10 g/mL insulin (Sigma-Aldrich GmbH). Erythrocyte differentiation was induced on the addition of 100 ng/mL SCF, 10^{-6} M hydrocortisone (Sigma-Aldrich GmbH), 5 ng/mL IL-3, and 3 IU/mL erythropoietin (Eprex; Janssen-Cilag). After 8 days of culture, erythroblasts were cocultured with MS-5 stromal cells in the presence of erythropoietin for 3 days. Finally, cells were cultured on MS-5 cells in the absence of cytokines until day 18. U0126 (5 μM; Biomol International LP) was added to the cells every 3 or 4 days to inhibit MEK1/2 activity. Cord blood was collected after informed consent was obtained in accordance with the Declaration of Helsinki. Protocols were approved by the ethics committee of the University Medical Center in Utrecht.

Colony-forming unit assay

Five hundred freshly isolated CD34+ cells or retrovirally transduced cells were plated in IMDM supplemented with 35.3% FCS, 44.4% Methocult medium (Stem Cell Technologies), β-mercaptoethanol (11.1 μM), penicillin (2.2 U/mL), streptomycin (2.2 μg/mL), and glutamine (0.44 mM). Colony-forming unit (CFU) assays were performed in the presence of SCF (50 ng/mL), FLT-3L (50 ng/mL), GM-CSF (0.1 nmol/L), IL-3 (0.1 nmol/L), and G-CSF (0.2 nmol/L). Colonies were scored after 12 days of culture.

Viral transduction of CD34+ cells

Bicistronic retroviral constructs were used to coexpress a 4-hydroxytamoxifen (4-OHT)–inducible active MEK1 mutant (MEK1:ER*) and enhanced green fluorescent protein (eGFP). Retrovirus was produced by transfection of the Phoenix-ampho packaging cell line by calcium-phosphate coprecipitation. After 2 weeks of selection with puromycin, cells were grown in a minimal amount of medium for 24 hours. Viral supernatants were collected and filtered through a 0.2-μm filter. CD34+ cells were transduced in 24-well dishes precoated with 1.25 μg/cm^2 recombinant human fibronectin fragment CH-296 (RetroNectin; Takara) overnight at 4°C. Transduction was performed by the addition of 0.5 mL viral supernatant to 0.5 mL cell suspension. Twenty-four hours after transduction, 0.7 mL medium was removed from the cells, and 0.5 mL fresh virus supernatant was added together with 0.5 mL medium.

Histochemical staining of hematopoietic cells

May-Grünwald-Giemsa staining was used to analyze maturation. Cytospins were prepared from 50 000 differentiating granulocytes. After fixation in methanol for 3 minutes, cytospins were stained in a 50% Eosin Methylene Blue solution according to May-Grünwald (Sigma-Aldrich GmbH) for 20 minutes, rinsed in water for 5 seconds, and stained with 10% Giemsa solution (Merck) for 15 minutes. Micrographs were acquired with an Axiostar plus microscope (Carl Zeiss) fitted with a 100×/1.3 NA EC Plan Neofluor oil objective using Immersol 518F oil (Carl Zeiss), a Canon Powershot G5 camera (Canon Nederland), and Canon Zoombrowser EX image acquisition software. Photoshop CS2 was used for image processing (Adobe Systems Benelux). Differentiated neutrophils were characterized as cells containing banded or segmented nuclei. A minimum of 100 cells per cytospin were counted in multiple randomly selected microscopy fields.

^{3}H-thymidine incorporation assays

CD34+ cells (n = 80 000) were incubated with 1 μCi (0.037 Bq)/mL ^{3}H-thymidine for 72 hours at day 8. The amount of ^{3}H-thymidine incorporated over a 3-day period was measured, and data were depicted as the percentage of ^{3}H-thymidine incorporation compared with control cells.

Single-cell proliferation assay

CD34+ progenitor cells ectopically expressing MEK1:ER* were seeded in Terasaki plates (Nunc) at a density of 1 cell/well in 20 μL IMDM containing SCF, FLT-3L, GM-CSF, IL-3, and G-CSF in the presence or absence of 20 nM 4-OHT. The wells were scored for colonies after 12 days of incubation.

Transwell migration assays

CD34+ cells, either retrovirally transduced to ectopically express MEK1:ER* or left untreated, were cultured in the presence of G-CSF to induce neutrophil differentiation. After 6 days of culture, cells ectopically expressing MEK1ER were washed and resuspended in culture medium containing G-CSF either in the presence or in the absence of 4-OHT for 16 hours. Subsequently, a transwell migration assay (Corning; pore size, 5 μm) was performed to determine the migratory behavior of granulocyte progenitors in response to conditioned MERK1:ER* medium or as a control culture medium with or without 4-OHT, which was added to the lower chamber.

Untreated control granulocyte progenitors (n = 50 000) from the same donor were added to the upper compartment, and the assays were performed for 4.5 hours at 37°C in the CO_2 incubator, after which time the percentage of migration was determined by fluorescence-activated cell sorter (FACS) analysis by using Flow count beads (Beckman Coulter).

Proliferation assays in MEK-conditioned media

$CD34^+$ cells, either retrovirally transduced to ectopically express MEK1:ER* or left untreated, were cultured in the presence of G-CSF to induce neutrophil differentiation. After 6 days of culture, cells ectopically expressing MEK1:ER* were washed and resuspended in culture medium containing G-CSF either in the presence or in the absence of 4-OHT for 16 hours to prepare "conditioned" medium. After 7 days of culture, 200 000 untreated control granulocyte progenitors from the same donor were resuspended in either conditioned medium or culture medium containing 4-OHT as a control in the presence of G-CSF and IL-3. Proliferation over a 3-day period was determined by cell counting.

Lactoferrin staining of hematopoietic cells

Neutrophil differentiation was analyzed by intracellular staining of lactoferrin. Cells were fixed in 100 μL 0.5% formaldehyde for 15 minutes at 37°C, after which time the cells were permeabilized in 900 μL ice-cold methanol for 30 minutes on ice. Cells were subsequently washed with PBS, resuspended in phycoerythrin-conjugated lactoferrin antibody (Immunotech), and incubated for 25 minutes. Cells were again washed, and FACS analysis was performed (FACSCalibur; Becton Dickinson).

Measurement of apoptosis

Apoptotic and necrotic cells were measured by staining with annexin V and propidium iodide (Alexis) according to the manufacturer's protocol.

Multiplex immunoassay

Neutrophil progenitors, ectopically expressing MEK:ER*, were cultured either in the absence or in the presence of 4-OHT for 12 hours before collecting supernatants. The IL-8 (Sanquin), CCL-2 (R&D Systems), and CCL-3 (R&D Systems) capture antibodies were covalently coupled to carboxylated polystyrene microspheres (Luminex Corporation).[34,35] To block any residual of interfering antibodies, the samples were diluted with 10% (vol/vol) normal rat and mouse serum (1:1 ratio; Rockland). Samples were run undiluted and diluted 1:50 in high-performance enzyme-linked immunoabsorbent assay buffer (Sanquin). Samples were measured, and blank values were subtracted from all readings.[34,35] Measurements and data analysis were performed by using the Bio-Plex system in combination with the Bio-Plex Manager software Version 4.0 with 5 parametric curve fitting (Bio-Rad).

Western blot analysis

Cells were lysed in Laemmli buffer (0.12 M Tris HCl pH 6.8, 4% SDS, 20% glycerol, 0.05 μg/μL bromophenol blue, and 35 mM β-mercaptoethanol) and boiled for 5 minutes. Equal amounts of lysate were analyzed by SDS–polyacrylamide gel electrophoresis. Proteins were transferred to Immobilon-P and incubated with blocking buffer (Tris-buffered saline/Tween-20) containing 5% low-fat milk for 1 hour before incubating with antibodies against Bcl-xL, p21Cip1, β-actin (all from Santa Cruz Biotechnology Inc) or ERK1/2 (Cell Signaling Technology Inc) overnight at 4°C. Before incubation with antibodies against phosphorylated ERK1/2 and phosphorylated p38 (both from Cell Signaling Technology Inc), blots were incubated in blocking buffer containing 5% BSA. Blots were subsequently incubated with peroxidase-conjugated secondary antibodies for 1 hour. Enhanced chemical luminescence was used as a detection method (Amersham Pharmacia).

RNA isolation

Neutrophil progenitors, ectopically expressing MEK1:ER*, were cultured either in the absence or in the presence of 4-OHT for 12 hours before lysing the cells in 1 mL Trizol (Invitrogen). Samples were subsequently incubated for 3 minutes at room temperature, 0.2 mL chloroform was added, vortexed and incubated for 3 minutes at room temperature, followed by 15 minutes of centrifugation at 8000 rpm at 4°C. Subsequently, 0.5 mL isopropanol was added to the aqueous phase and incubated for 30 minutes at −20°C. Samples were centrifuged at 14 000 rpm for 10 minutes at 4°C. The pellet was washed with 70% ethanol and dissolved in water. DNAse treatment and purification was performed with the use of RNeasy kit (QIAGEN).

Microarray analysis

cRNA (500 ng) coupled to Cy3 and Cy5 fluorophores (Amersham) was hybridized onto Codelink activated slides (GE Healthcare) containing the Operon Human Genome Oligo Set V2. Hybridized slides were scanned on an Agilent scanner (G2565AA) at 100% laser power, 100% PMT. After data extraction with Imagene 7.5 (BioDiscovery), printtip Loess normalization was performed. Data were analyzed with ANOVA (R2.2.1/MAANOVA Version 0.98-3; www.rproject.org/). In a fixed effect analysis, sample, array, and dye effects were modeled. *P* values were determined by a permutation F2 test, in which residuals were shuffled 5000 times globally. Genes with *P* values less than .05 after family-wise error correction were considered significantly changed. Visualization and cluster analysis were done with GeneSpring 7.2 (Agilent). Microarray data have been deposited with ArrayExpress under accession no. E-TABM-754.

Statistics

An independent sample *t* test for was performed to compare the differences in proliferation, differentiation, and colony-forming capacity. A *P* value of .05 or less was considered significant (*).

Results

MEK-ERK signaling is required for expansion of $CD34^+$-derived neutrophil progenitors

To determine whether the MEK-ERK signaling pathway plays a critical role in regulation of neutrophil development, an ex vivo differentiation system was used. Human $CD34^+$ hematopoietic progenitor cells, isolated from umbilical cord blood, were cultured in the presence of G-CSF to induce neutrophil differentiation.[36] Cells were cultured either in the absence or in the presence of U0126, a specific pharmacologic inhibitor of MEK1/2, and differences in expansion and survival were analyzed. The specificity of U0126 was confirmed by its ability to inhibit the phosphorylation of ERK1/2 in $CD34^+$ cells but not p38MAPK phosphorylation (Figure 1A). U0126 significantly inhibited progenitor expansion during neutrophil differentiation as determined by both cell counting and ^{3}H-thymidine incorporation assays. (Figure 1B-C). To determine whether U0126-mediated inhibition of expansion was caused by increased levels of apoptosis, the percentage of annexin V–positive cells was analyzed. Inhibition of ERK MAPK activity did not significantly alter the levels of annexin V–positive cells during the first 2 weeks; however, an increase in apoptosis was observed after 14 days of neutrophil differentiation (Figure 1D).

To address the question as to whether MEK-ERK activity also plays a similar role in regulating the expansion of other nonmyeloid lineages, $CD34^+$ progenitors were differentiated toward erythrocytes for 18 days either in the presence or in the absence of U0126, and differences in expansion, survival, and differentiation were analyzed. In contrast to neutrophil development, the addition of U0126 completely abrogated expansion and subsequent differentiation of $CD34^+$ erythrocyte progenitors (Figure 2A-B), because of induction of apoptosis (Figure 2C). This was found to correlate with abrogation of antiapoptotic Bcl-xL levels in $CD34^+$ cells treated with U0126 (Figure 2D).

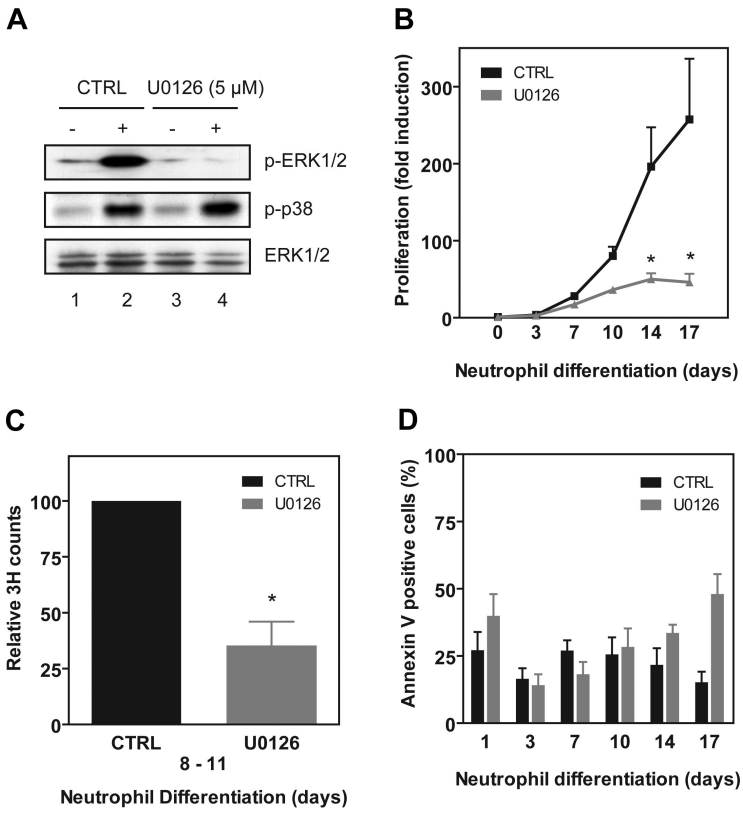

Figure 1. MEK-ERK activation is required for expansion of hematopoietic progenitor cells. (A) CD34+ cells were starved overnight in the absence of cytokines and in the presence of 0.5% FCS. Cells were left untreated (lanes 1-2) or treated with 5 μM U0126 (lanes 3-4) for 1 hour before stimulation with G-CSF (lanes 2,4) for 15 minutes. Protein lysates were prepared, and Western blot analysis was performed with an antibody against phosphorylated ERK1/2 and phosphorylated p38MAPK, and as a control for equal loading an antibody against total ERK1/2. (B-C) CD34+ cells were cultured for 17 days in the presence of G-CSF to induce neutrophil differentiation in the absence or presence of 5 μM U0126. Expansion was determined by (B) counting the trypan blue–negative cells or by (C) performing ^{3}H-thymidine incorporation assays. Data were depicted as a ratio between control and cells treated with U0126. (D) During the 17-day culture period the percentage of apoptotic cells was determined by annexin V staining. Results are presented as means of 4 independent experiments. Error bars represent SEMs.

Together, these results show that MEK-ERK activity is essential for survival and development of erythroid progenitors and for expansion of neutrophil progenitors.

MAPK activity is not essential for neutrophil differentiation of hematopoietic progenitor cells

Having established the effects of MEK inhibition on neutrophil progenitor expansion, we were interested to determine whether MEK-ERK also plays a role in regulating neutrophil differentiation. CD34+ cells, isolated from umbilical cord blood, were differentiated toward neutrophils for 17 days either in the presence or in the absence of U0126. After 14 and 17 days of culture, cytospins were prepared, and the morphology of the differentiating granulocytes was analyzed by May-Grünwald-Giemsa staining. Treatment of hematopoietic CD34+ cells with U0126 did not alter the total percentage of differentiated neutrophils (Figure 3A-B). However, because U0126 inhibited progenitor expansion during neutrophil development, the absolute numbers of mature neutrophils after 14 and 17 days of culture were reduced (Figure 3C). Furthermore, flow cytometric analysis was performed to support the morphologic observations and showed that treatment with U0126 did not affect the expression of lactoferrin, a neutrophil-specific granule protein (Figure 3D).

To assess the development of single hematopoietic progenitors, CD34+ cells were plated in CFU assays, either in the presence or in the absence of U0126, and colony formation was analyzed after 12 days. Similarly, inhibition of MEK-ERK signaling did not significantly affect the capacity of the GM colony formation of hematopoietic progenitor cells (Figure 3E). However, absolute cell numbers of total GM colonies were significantly decreased in the presence of U0126, indicating that MEK-ERK activity is indeed required for the expansion of hematopoietic progenitors during myeloid development (Figure 3F). Together these results show that MEK-ERK activity, although essential for expansion, is not required for differentiation of CD34+ progenitor cells during neutrophil development.

Conditional activation of MEK1 promotes expansion of hematopoietic progenitors during neutrophil differentiation

To further validate and extend the results obtained with the MEK1/2 pharmacologic inhibitor, CD34+ cells, cultured in the presence of G-CSF, were retrovirally transduced to ectopically express MEK1:ER*.[37] In the absence of 4-OHT, activation of MEK1:ER* is inhibited by heat-shock and chaperone proteins that associate with the fused ER hormone–binding domain. On the addition of 4-OHT, these proteins dissociate, resulting in the release of active MEK1. Three days after transduction, eGFP-positive cells were sorted from the nontransduced cells and resuspended in culture medium containing G-CSF in the presence or absence of 20 nM 4-OHT. Activation of ERK1/2 in CD34+ cells transduced with MEK1:ER* on 4-OHT stimulation was confirmed by Western blotting (Figure 4A). MEK1 activation resulted in significant expansion of progenitor cells between day 3 and day 7; however, prolonged MEK1 activation prevented neutrophil differentiation because of massive apoptosis (Figure 4B-C).

In addition, to assess the expansion of single hematopoietic progenitors, CD34+ cells transduced with MEK1:ER* were seeded in Terasaki plates at a density of 1 cell per well in culture medium containing G-CSF in the presence or absence of 20 nM 4-OHT. After 12 days, positive wells were scored. MEK1 activation resulted in a significant increase in the proliferative capacity of CD34+ cells (Figure 4D). To further understand how MEK-ERK regulates progenitor numbers over time, CD34+ progenitor cells transduced with MEK1:ER* were differentiated toward neutrophils

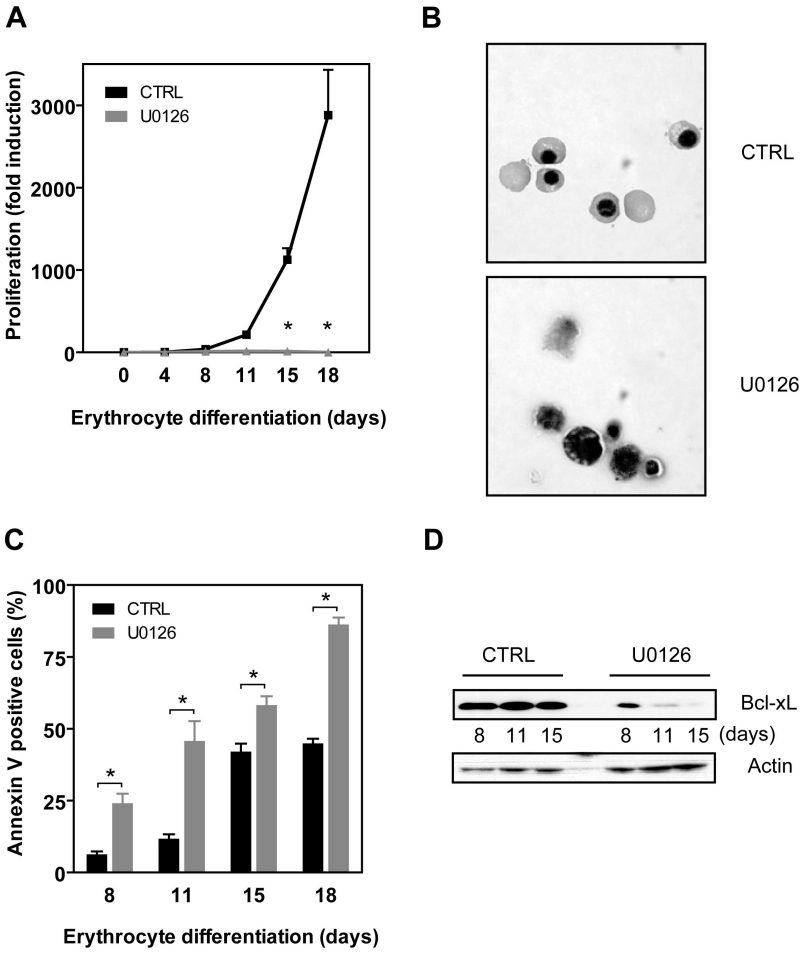

Figure 2. MEK activity is essential for the survival of hematopoietic progenitors during erythropoiesis. CD34$^+$ progenitors were differentiated toward erythrocytes for 18 days either in the presence or in the absence of 5 μM U0126. (A) Expansion was determined by counting the trypan blue–negative cells. (B) After 18 days of culture, cytospins were prepared and stained with May-Grünwald-Giemsa solution. (C) During the 18-day culture period, the percentage of apoptotic cells was determined by annexin V staining. Results are presented as means of 4 independent experiments. Error bars represent SEMs. (D) Protein lysates were prepared from CD34$^+$ cells differentiated toward erythrocytes for 8, 11, and 15 days in the presence or absence of 5 μM U0126. Western blot analysis was performed with an antibody against Bcl-xL (top) or β-actin (bottom) as a control for equal loading. Similar results were obtained in 3 independent experiments.

in the presence or absence of 4-OHT. After either 3 or 7 days of culture, cells were plated in CFU assays in the absence of 4-OHT, and colony formation was further analyzed after 12 days of culture. Remarkably, GM colony formation of CD34$^+$ cells, ectopically expressing MEK1:ER* that had been cultured in the presence of 4-OHT for 3 and 7 days, was significantly decreased compared with controls (Figure 4E). These data suggest that constitutive activation of MEK-ERK is not sufficient to overcome the natural loss of colony-forming capacity or to retain the early immature progenitor phenotype in the presence of lineage-directing cytokines, such as G-CSF. Together, these results suggest that tight control of MEK-ERK activity is an important factor in regulating the balance between expansion and survival of hematopoietic progenitors during neutrophil differentiation.

Conditional activation of MEK1 does not affect terminal differentiation of neutrophil progenitors

To further evaluate the effect of MEK1 on neutrophil development, CD34$^+$ cells were transduced with MEK1:ER*, sorted from nontransduced cells, and differentiated to neutrophils in the presence or absence of 4-OHT. After 3 days of culture, cells were washed and resuspended in culture medium containing G-CSF. After 14 and 17 days of differentiation, cytospins were prepared, and the morphology of the cells was analyzed. Although prolonged activation of MEK1 prevented neutrophil differentiation because of induction of cell death, the addition of 4-OHT for 3 days resulted in an increase in progenitor cell numbers, which thereafter differentiated normally to mature neutrophils (Figure 5A). However, because MEK1 activation resulted in significant expansion of progenitor cells between day 3 and day 7, the absolute numbers of mature neutrophils after 17 days of culture were also increased (Figure 5B). In addition, flow cytometric analysis was also performed to support the morphologic observations, and conditional MEK1 activation had no significant effect on the expression of lactoferrin, a neutrophil-specific granule protein (Figure 5C).

To assess the development of single hematopoietic progenitors, CD34$^+$ cells transduced with MEK1:ER* were plated in CFU assays, either in the presence or in the absence of 4-OHT, and colony formation was analyzed after 12 days. Similarly, activation of MEK1 by the addition of 4-OHT did not affect the GM colony formation capacity of hematopoietic progenitor cells (Figure 5D). Taken together, although found to be an essential regulator of expansion and survival of myeloid progenitors, these results show that MEK-ERK signaling does not modulate terminal differentiation of neutrophil progenitors.

Analysis of differential gene expression in response to MEK1 activation

Taken together, these results show that tight control of MEK-ERK signaling plays an important role in the regulation of progenitor expansion and survival. To identify changes in gene expression that might underlie these observations, a microarray analysis was performed. CD34$^+$ cells retrovirally transduced to ectopically express MEK1:ER* were cultured in the presence of

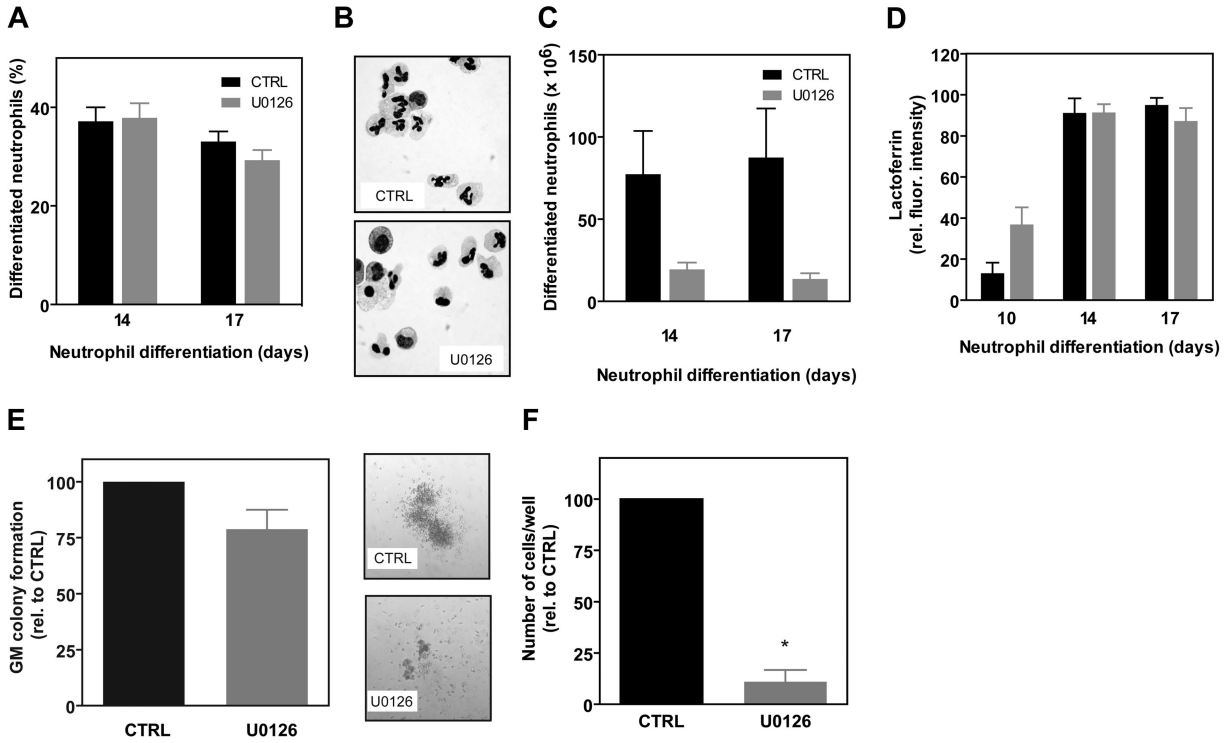

Figure 3. ERK MAPK activity is not required for myeloid differentiation of hematopoietic progenitor cells. (A-C) CD34+ cells were cultured either in the presence of G-CSF to induce neutrophil differentiation or in the absence of 5 μM U0126. (B) After 14 and 17 days of culture, cytospins were prepared and stained with May-Grünwald-Giemsa solution. Data were expressed as either (A) the percentage of differentiated cells or (C) the absolute cell numbers. Data were expressed as the percentage of differentiated neutrophils. (D) Lactoferrin expression was analyzed by FACS to determine neutrophil development. (E) Freshly isolated CD34+ progenitor cells were plated in CFU assays, either in the presence or in the absence of 5 μM U0126, and colony formation was analyzed after 12 days. (F) Freshly isolated CD34+ progenitor cells were plated in CFU assays, either in the presence or in the absence of 5 μM U0126, and absolute cell numbers of total GM colonies were analyzed after 12 days. Results are presented as means of 4 independent experiments. Error bars represent SEMs.

G-CSF and IL-3 to induce neutrophil differentiation. After 6 days of culture, cells were starved overnight in the absence of cytokines. Cells were subsequently stimulated with 20 nM 4-OHT or carrier for 12 hours before RNA was isolated. Results obtained from 4 independent donors were subjected to both a filter query and statistical analysis, allowing only those datasets to pass that were increased or decreased and showed a fold change of 2 or greater in all 4 independent sets compared with the carrier control. The resulting gene set representing 181 differentially expressed genes in response to MEK1 activation were classed into functional groups that are shown in Table 1 and supplemental Figure 1 (available on the *Blood* website; see the Supplemental Materials link at the top of the online article). The number of genes induced by MEK1 activation is much larger than the number of genes that show down-regulation, consistent with MAPK being primarily an inducer of transcription. The transcripts c-myc, c-fos, p21CIP1, cyclin D1, and cyclin D3, all of which are involved in regulation of cell cycle, show strong up-regulation onMEK1 activation. Interestingly, transcripts coding for hematopoietic cytokines and growth factors, such as IL-8, IL-1β, and various members of the CCL and CXCL family, were strongly up-regulated in response to MEK1 activation (Table 1). To validate changes in gene expression, a subset of the identified MEK1 target genes was verified by using Luminex multiplex analysis (Figure 6A). IL-8, CCL2, and CCL3 showed dramatic up-regulation in response to MEK1. Furthermore, whole-cell lysates prepared of MEK1:ER*-transduced cells stimulated with 0, 20, or 100 nM 4-OHT were used for detection of p21Cip1, which also showed significant up-regulation in response to MEK1 activation (Figure 6B). These data clearly show that conditional activation of MEK1 in CD34+ progenitor cells results in changes in the expression of a variety of proliferation-modulating genes, consistent with the MEK-ERK module being an important regulator of progenitor expansion.

To determine whether MEK1-induced expression of hematopoietic cytokines and chemokines is implicated in the regulation of either expansion or migration of myeloid progenitors, CD34+ cells were transduced with MEK1:ER* or left untreated and cultured in the presence of G-CSF to induce neutrophil differentiation. After 6 days of culture, cells ectopically expressing MEK1:ER* were washed and resuspended in culture medium containing G-CSF either in the presence or in the absence of 4-OHT for 16 hours to prepare conditioned medium. Subsequently, untreated control neutrophil progenitors from the same donor were resuspended in either conditioned medium or culture medium containing 4-OHT as a control in the presence of G-CSF and IL-3, and differences in progenitor expansion were analyzed over a 3-day period by cell counting. Proliferation of cells in conditioned medium was increased compared with control medium, indicating that MEK1-induced cytokines and chemokines can modulate the expansion of hematopoietic progenitors during myeloid development (Figure 6C). Furthermore, to determine whether there was a change in the migratory behavior of granulocyte progenitors in response to conditioned MEK1:ER*-conditioned medium, a transwell migration assay was performed with untreated control neutrophil progenitors from the same donor. The addition of conditioned medium obtained from cells expressing active MEK1 resulted in increased

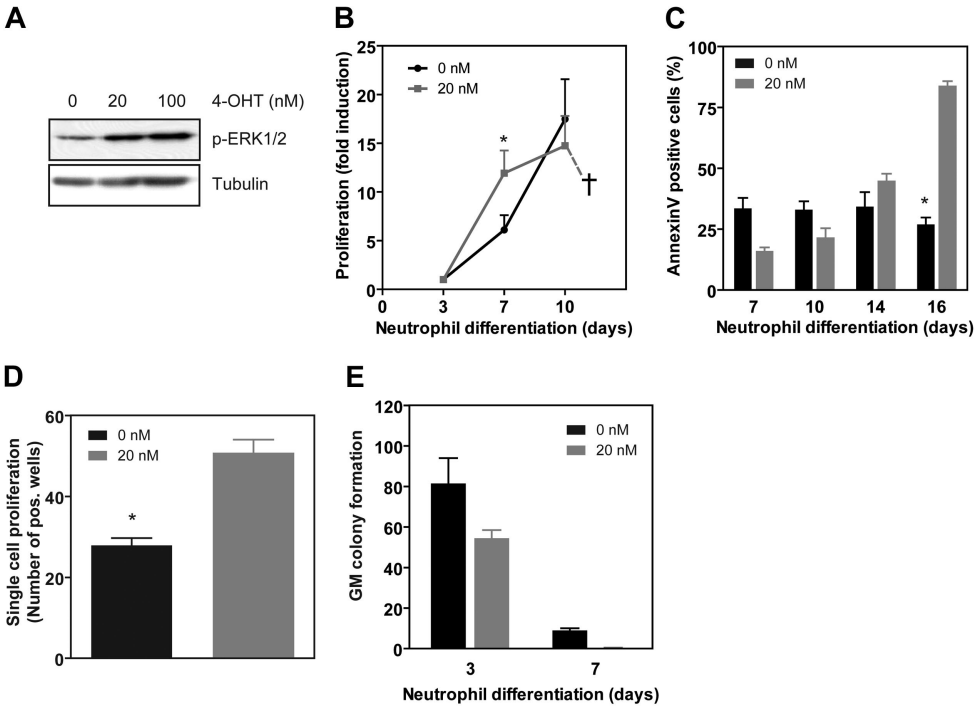

Figure 4. Conditional activation of MEK1 promotes expansion of myeloid progenitors. (A) Protein lysates were prepared from CD34$^+$ cells transduced with MEK:ER* and treated with solvent, 20 or 100 nM of 4-OHT for 30 minutes. Western blot analysis was performed with an antibody against phosphorylated ERK1/2 or tubulin as a control for equal loading. Similar results were obtained in 4 independent experiments. (B) CD34$^+$ cells were transduced with MEK1:ER*, sorted by FACS from the nontransduced cells, and differentiated to neutrophils in the presence or absence of 20 nM 4-OHT. Expansion was determined by counting the trypan blue–negative cells. (C) CD34$^+$ cells were transduced with MEK1:ER*, sorted by FACS from the nontransduced cells, and differentiated to neutrophils in the presence or absence of 20 nM 4-OHT. During the 17-day culture period the percentage of apoptotic cells was determined by annexin V staining. (D) CD34$^+$ cells, transduced with MEK:ER and sorted by FACS, were seeded in 60-well plates at a density of 1 cell per well in normal culture medium containing G-CSF in the presence or absence of 20 nM 4-OHT. After 12 days, wells with colonies were scored. (E) CD34$^+$ progenitor cells transduced with MEK1:ER* were differentiated toward neutrophils in the presence or absence of 4-OHT. After either 3 and 7 days of culture, cells were plated in CFU assays in the absence of 4-OHT, and colony formation was analyzed after 12 days of culture. Results are presented as means of 4 independent experiments. Error bars represent SEMs.

migration of neutrophil progenitors compared with control medium (Figure 6D). Taken together, these data suggest that the MEK-ERK pathway may, at least in part, be involved in the regulation of expansion and migration of hematopoietic progenitor during neutrophil development through modulating the expression of hematopoietic cytokines and chemokines.

Discussion

In this study, we have investigated the role of the MEK-ERK MAPK signaling pathway in CD34$^+$ hematopoietic progenitor function, focusing on neutrophil development. The data presented here show that ERK MAPK activity is required for the initial expansion of CD34$^+$ hematopoietic progenitors during both neutrophil and erythroid differentiation. Subsequent microarray analysis of CD34$^+$ cells showed that activation of MEK1 resulted in changes in expression of a variety of genes encoding hematopoietic cytokines and growth factors. In addition, we observed up-regulation of genes involved in regulating cell-cycle progression, including c-myc, c-fos, cyclin D1, and cyclin D3.

During the G_1 phase of the cell cycle, D-type cyclins assemble with cyclin-dependent kinases (CDKs) facilitating cell-cycle progression through phosphorylation of several key substrates, such as retinoblastoma protein.[38] Previously, it has been shown that activation of the ERK pathway in epithelial cells by expression of an conditionally active Raf1 mutant resulted in increased cyclin D1 expression, whereas blocking ERK activity by expressing a dominant-negative form of MEK led to decreased cyclin D1 expression.[39] However, cyclin D1 was not induced immediately after growth factor stimulation, but only after sustained ERK activity, suggesting that this was not directly because of ERK activation.[40] Candidates potentially mediating ERK transcriptional induction of cyclin D1 gene are the c-fos and c-myc transcription factors. Daksis et al[41] and Seth et al[42] have demonstrated that direct phosphorylation of c-myc on serine 62 by ERK MAPK enhanced its stability, resulting in subsequent transcriptional induction of the cyclin D1 gene. Furthermore, it has been shown that activated ERK can phosphorylate preexisting transcription factors, such as Elk-1, involved in the up-regulation of immediate-early gene c-fos. Increased expression of many other genes through up-regulation of c-fos, including fra-1, ultimately results in expression of cyclin D1.[43] Thus, expression of immediate-early genes has been shown to regulate subsequent induction of delayed early genes, which include cyclin D1. Because we also observed increased expression of c-fos and c-myc, it is likely that the MEK-ERK pathway regulates expansion of CD34$^+$ progenitors through this mechanism, resulting in up-regulation of cyclin D1 and subsequent assembly of active cyclin D–CDK complexes, facilitating cell-cycle progression.

Interestingly, Furukawa et al[44] have demonstrated that expression of cell-cycle control genes, including D-type cyclins, is differentially regulated in a lineage-specific manner during hematopoiesis, suggesting that these genes not only are involved in cell-cycle regulation but also play a role in lineage-restricted functions of hematopoietic cells. Lineage-specific changes, for

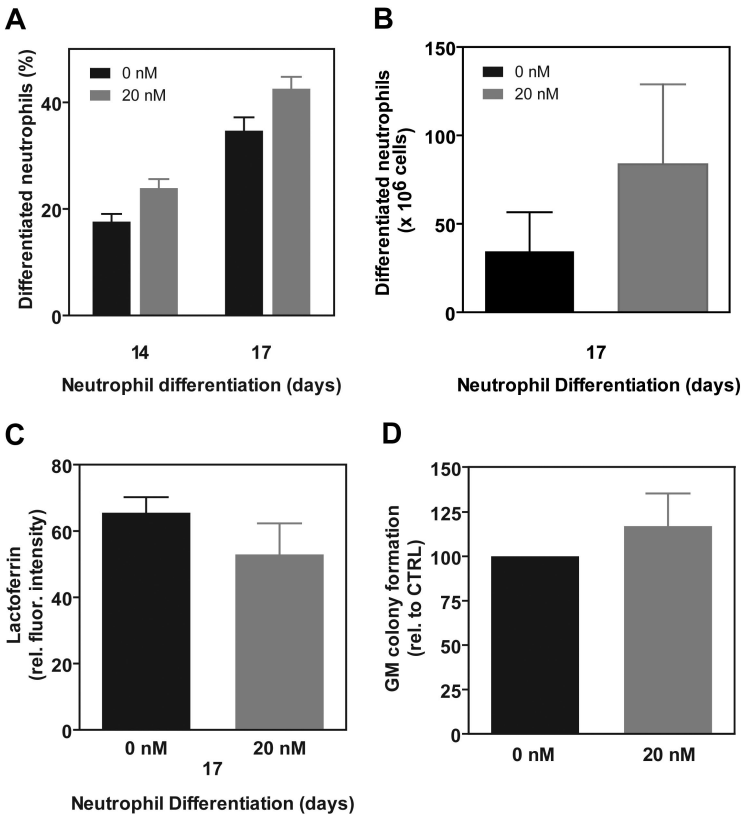

Figure 5. Conditional activation of MEK1 does not affect terminal differentiation of neutrophil progenitors. (A-B) CD34+ cells were transduced with MEK1:ER*, sorted by FACS from the nontransduced cells, and differentiated to neutrophils in the presence or absence of 4-OHT. After 3 days of culture, cells were washed and resuspended in normal culture medium containing G-CSF. After 14 and 17 days of differentiation, cytospins were prepared, and the morphology of the cells was analyzed by May-Grünwald-Giemsa staining. Data were expressed as either (A) the percentage of differentiated cells or as (B) the absolute cell numbers. Data were expressed as the percentage of differentiated neutrophils. (C) Lactoferrin expression was analyzed by FACS to determine neutrophil development. (D) CD34+ cells, transduced with MEK1:ER*, were sorted by FACS from the nontransduced cells and plated in CFU assays, either in the presence or absence of 20 nM 4-OHT, and colony formation was analyzed after 12 days. Results are presented as means of 4 independent experiments. Error bars represent SEMs.

Table 1. Genes in response to MEK1 activation

Group/gene	Fold change	Definition
Cytokine–cytokine receptor interaction		
IL8	31.67	Interleukin-8
IL1B	19.99	Interleukin-1β
CCL4	18.89	Chemokine (C-C motif) ligand 4
CCL2	17.88	Chemokine (C-C motif) ligand 2
CCL7	9.97	Chemokine (C-C motif) ligand 7
CXCL3	5.73	Chemokine (C-X-C motif) ligand 3
CXCL2	4.2	Chemokine (C-X-C motif) ligand 2
IL1A	2.78	Interleukin-Iα
CCL5	2.76	Chemokine (C-C motif) ligand 5
IFNGR2	2.61	Interferon γ receptor 2
TGFB1	2.25	Transforming growth factor β 1
G-CSFR	0.42	Granulocyte colony-stimulating factor receptor precursor
CD70	0.48	CD70 molecule
NGFR	0.5	Nerve growth factor receptor
Cell cycle and apoptosis		
EGR1	9.76	Early growth response protein 1
p21Cipl	3.63	Cyclin-dependent kinase inhibitor 1A
CCND1	2.96	Cyclin D1
BIRC3	2.85	Baculoviral AP repeat-containing 3
NFKBIA	2.63	NFκB inhibitor α
CCND3	2.41	Cyclin D3
MYC	2.39	Myc transcription factor
FOS	2.15	Fos gene family transcription factor
NOXA	2.03	Bol-2 protein family member Noxa
XRCC3	0.45	DNA-repair protein XRCCI
GADD45B	0.54	Growth arrest and DNA-damage–inducible β

example, include sustained elevation of cdc2 and cyclin A during erythrocyte differentiation and selective induction of cyclin D1 in myeloid progenitors. However, little is known about the function of cyclin D1 in terminally differentiated myeloid cells. Zwijsen et al[45] reported that cyclin D1 directly interacts with the estrogen receptor and enhances its binding to estrogen-responsive elements, thereby activating estrogen-mediated transcription. Through a similar mechanism, cyclin D1 may thus be involved in the regulation of myeloid-specific genes. Our data support the idea that the MEK-ERK signaling module plays a critical role in regulation of expansion of myeloid precursor cells through up-regulation of genes that are required for cell-cycle progression, such as cyclin D1. However, maturation of granulocyte progenitors was unaffected, suggesting that cyclin D1 is acting predominantly as a regulator of proliferation in CD34+ progenitors.

Besides transcriptional induction of cyclin D1, various inhibitors of signaling were up-regulated, such as MAPK phosphatases DUSP2, DUSP5, and DUSP6, a subclass of protein tyrosine phosphatases that specifically dephosphorylate threonine and tyrosine residues on MAPKs and are thought to be part of a negative feedback loop.[46] Moreover, p21Cip1 was found to be up-regulated by MEK1 activation in CD34+ cells (Figure 6B). p21Cip1 is best known for its function as a cell-cycle inhibitor and was shown to associate and inhibit cyclin-CDK complexes during the G_1 phase of the cell cycle.[47] Interestingly, by using epithelial cells expressing a conditionally active Raf mutant, it was shown that strong and sustained Raf activity throughout the G_1 phase of the cell cycle triggered cell-cycle arrest by inducing massive expression of the cell-cycle inhibitor p21Cip1, whereas modest activation resulted in enhanced cell-cycle entry.[48,49] It is possible that because of CD34+ progenitors the p21Cip1 expression observed at low 4-OHT concentrations could also be partly responsible for increased proliferation.

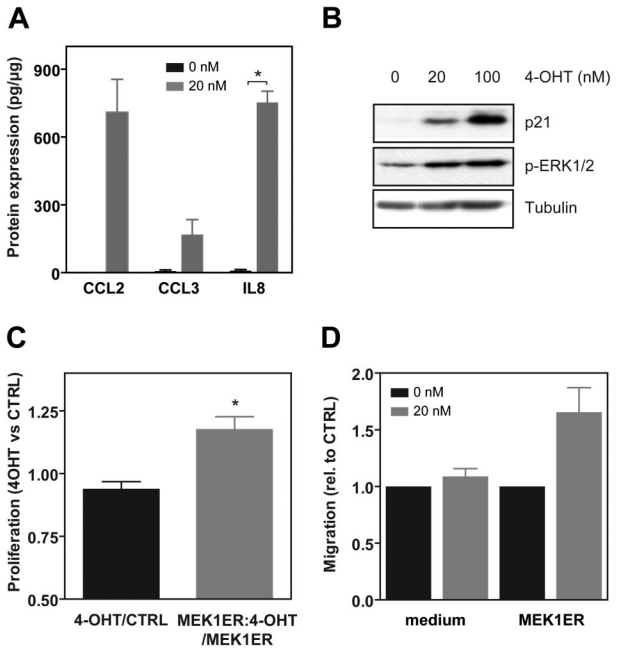

Figure 6. Differential expression of MEK1 target genes. (A) CD34$^+$ hematopoietic progenitor cells, transduced with MEK1:ER*, were cultured in the presence of G-CSF to induce neutrophil differentiation. Two days after transduction, eGFP-positive cells were separated from the nontransduced cells by FACS and resuspended in culture medium containing G-CSF and IL-3. After 6 days of differentiation, cells were washed twice with PBS and starved overnight in the absence of cytokines and in the presence of 0.5% FCS. Subsequently, cells were incubated with 0 or 20 nM 4-OHT for 12 hours; supernatants were collected; and IL-8, CCL2, and CCL3 protein expression was determined by using Luminex multiplex analysis as described in "Analysis of differential gene expression in response to MEK1 activation." Results are presented as the means of 2 or 3 independent experiments. Error bars represent SEMs. (B) Protein lysates were prepared from CD34$^+$ cells transduced with MEK:ER* and treated with solvent, 20 or 100 nM of 4-OHT overnight. Western blot analysis was performed with an antibody against p21Cip1, phosphorylated ERK1/2, or tubulin as a control for equal loading. (C) CD34$^+$ cells, either transduced with MEK1:ER* or left untreated, were differentiated toward neutrophils in the presence of G-CSF. After 6 days of culture, cells ectopically expressing MEK1:ER* were washed and resuspended in culture medium either in the presence or absence of 4-OHT to prepare conditioned medium. After 7 days of culture, untreated control granulocyte progenitors from the same donor were resuspended in either conditioned medium or culture medium containing 4-OHT as a control. Proliferation over a 3-day period was determined by cell counting. (D) CD34$^+$ cells, either transduced with MEK1:ER* or left untreated, were cultured in the presence of G-CSF to induce neutrophil differentiation. After 6 days of culture, cells ectopically expressing MEK1ER were washed and resuspended in culture medium either in the presence or in the absence of 4-OHT. A transwell migration was performed with untreated control granulocyte progenitors from the same donor in response to conditioned MEK1ER medium or as a control culture medium with or without 4-OHT. The assays were performed for 4.5 hours, after which the percentage of migration was determined by FACS analysis. Data were expressed as an average of 4 individual experiments. Error bars represent SEMs.

Several studies have provided evidence that the MEK-ERK signaling module is often constitutively activated in acute myeloid leukemia (AML).[50] For example, Morgan et al[51] have shown activation of the MEK-ERK pathway in 9 of 14 AML cell lines studied, suggesting that aberrant activation of ERK kinases is indeed contributing to the abnormal cell growth in acute leukemias. Similarly, expression of a constitutively active form of Raf kinase in hematopoietic cell lines results in MEK activation and growth factor independence.[52] These properties make the MEK-ERK signaling module a potential target for the molecular therapy in AML, and various studies have shown the beneficial effects of chemotherapeutic agents that inhibit MEK-ERK activation in inducing growth suppression and apoptosis of AML cells.[51,53] Interestingly, our data also show that modulating the duration and extent of ERK activation is essential in regulating the balance between expansion and survival of hematopoietic progenitors during neutrophil differentiation. Inducible activation of MEK1 in CD34$^+$ cells through the addition of 4-OHT resulted in dramatic progenitor expansion during the first 3 days. However, in contrast to the effects of constitutive MEK activation in AML blast cells, prolonged MEK1 activation prevented neutrophil differentiation because of the induction of cell death. Together, this suggests that, although MEK-ERK signaling may indeed be a critical factor in the generation of myeloid leukemia, additional mechanisms are required for cellular transformation to occur. Thus, dysregulation of MEK-ERK signaling alone is insufficient for CD34$^+$ progenitor transformation, and cells are "culled" if this signaling module is inappropriately activated.

In addition to down-regulation of many antiproliferative genes, the ERK MAPK pathway is involved in the regulation of apoptosis through controlling expression of apoptotic regulatory molecules, including Bad, Bim, Mcl-1, caspase 9.[8] Our results have also shown that ERK MAPK activity is essential for survival of erythroid CD34$^+$ progenitors. Inhibition of ERK activity prevented erythropoiesis because of the induction of apoptosis in CD34$^+$ cells, which was found to correlate with a dramatic decrease in antiapoptotic Bcl-xL levels. In contrast, our results also show that MEK-ERK activity, although essential for expansion, is not required for the development of CD34$^+$ progenitor cells during neutrophil development. Although it is beyond the scope of this article to describe the lineage-specific effects of the MEK-ERK pathway, these results suggest that the MEK-ERK pathway plays a specific and differential role in regulating the development of myeloid and erythroid lineages.

Although our results have shown that MEK-ERK pathway is not essential for maturation of neutrophil progenitors, many of the 180 differentially expressed genes in response to MEK1 activation were genes coding for hematopoietic cytokines and chemokines, such as IL-8, IL-1β, and various members of the CCL and CXCL family. Computational analysis of the IL-8, IL-1β, CCL2, CCL-3, and CCL-7 genes that used the MAPPER Search Engine (http://mapper.chip.org) indeed identified putative transcription factor binding sites for MEK-ERK–induced transcriptional regulators, including the AP-1 transcription factor complex. Chemokines are involved in migration, homing, mobilization, proliferation, and survival of hematopoietic stem cells and hematopoietic progenitor cells, and they are also implicated in the development of malignancies.[54-56] It is plausible that they act as autocrine growth factors to activate signaling pathways implicated in the regulation of progenitor survival and proliferation.[57,58] For example, autocrine stimulation of the IL-8 receptor pathway has already been shown in human myeloid and lymphoid leukemia.[59] Furthermore, IL-8 in synergy with M-CSF was found to promote the clonogenic potential of CD34$^+$ cells, which involved an autocrine regulatory loop.[60] Our data further suggest that cytokine and chemokine production through MEK-ERK activation could contribute to hematopoietic homeostasis by regulating hematopoietic progenitor cell function during neutrophil development (Figure 6).

Taken together, we have identified a novel role for MEK-ERK signaling in regulating the balance between proliferation and apoptosis of CD34$^+$-derived neutrophil progenitor cells. This indicates the need for tight control of this signaling module because aberrant MEK-ERK activation could result in the development of bone marrow failure or hematologic malignancies.

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Authorship

Contribution: C.R.G., M.B., and M.J.K.G.K. performed experiments; C.R.G., M.B., M.J.K.G.K., E.V., and P.J.C. analyzed results; C.R.G. and M.B. made the figures; C.R.G., M.B., F.C.P.H., E.V., and P.J.C. designed the research; and C.R.G., M.B., and P.J.C. wrote the paper.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

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