MicroRNA-34a Inhibits Cell Proliferation by Repressing Mitogen-Activated Protein Kinase Kinase 1 during Megakaryocytic Differentiation of K562 Cells[S]

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ABSTRACT

Phorbol 12-myristate 13-acetate (PMA) induces megakaryocytic differentiation of the human chronic myelocytic leukemia cell line K562. We examined the potential regulatory role of microRNAs (miRNAs) in this process. Genome-wide expression profiling identified 21 miRNAs (miRs) that were induced by the treatment of K562 cells with PMA. Among them, the expression of miR-34a, miR-221, and miR-222 was induced in the early stages and maintained throughout the late stages of differentiation. Cell signaling analysis showed that the activation of extracellular signal-regulated protein kinase (ERK) in response to PMA strongly induced miR-34a expression by transactivation via the activator protein-1 binding site in the upstream region of the miR-34a gene. Reporter gene assays identified mitogen-activated protein kinase kinase 1 (MEK1) as a direct target of miR-34a and c-fos as a direct target of miR-221/222. Although overexpression of the three miRNAs had little effect on cell differentiation, overexpression of miR-34a significantly repressed the proliferation of K562 cells with a concomitant reduction in MEK1 protein expression. Conversely, a locked nucleic acid probe against miR-34a significantly enhanced the proliferation of PMA-treated K562 cells. Taken together, the results show that PMA activates the MEK-ERK pathway and strongly induces miRNA-34a expression, which in turn inhibits cell proliferation by repressing the expression of MEK1. Thus, the results highlight an important regulatory role for miR-34a in the process of megakaryocytic differentiation, especially in the arrest of cell growth, which is a prerequisite for cells to enter differentiation.

Hematopoietic differentiation is a complex, but highly ordered, process. The generation of mature blood cells involves the progressive differentiation of stem cells and multipotent progenitor cells into committed cells of various lineages. Cell lineage is thought to be regulated at the level of transcription, with lineage-specific transcription factors driving specific cell fates. The human chronic myelocytic lymphoma cell line K562 is widely used as a model for the study of hematopoietic differentiation (Lam et al., 2000; Ishiko et al., 2005). K562 cells are pluripotent hematopoietic cells that undergo either erythrocytic or megakaryocytic differentiation depending on the stimulus. Phorbol esters such as phorbol 12-myristate 13-acetate (PMA) induce megakaryocytic differentiation in K562 cells through activation of the protein kinase C (PKC) pathway (Shelly et al., 1998; Kim et al., 2001; Dorsey et al., 2002; Pettiford and Herbst, 2003). The PMA-induced differentiation process involves the marked arrest of cell growth, changes in cell morphology and adhesive properties, and the expression of specific markers of megakaryocytes such as the CD41, CD61, and CD10 surface antigens (Belhacène et al., 1998; Dorsey et al., 2002; Jacquel et al., 2006). In K562 cells, sustained activation of the mitogen-activated pro-

ABBREVIATIONS: PMA, phorbol 12-myristate 13-acetate; MEK, mitogen-activated protein kinase kinase; PKC, protein kinase C; miRNA (miR), microRNA; ERK, extracellular signal-regulated protein kinase; p-ERK, phospho-ERK; MAPK, mitogen-activated protein kinase; UTR, untranslated region; DMSO, dimethyl sulfoxide; RT, reverse transcription; PCR, polymerase chain reaction; qRT-PCR, quantitative real-time PCR; 5′ RACE, rapid amplification of 5′-cDNA ends; LNA, locked nucleic acid; U0126, 1.4-diamino-2,3-dicyano-1,4-bis[2-amino-phenylthio]butadiene; ANOVA, analysis of variance; AP-1, activator protein-1; C_t, threshold cycle; snRNA, small nuclear RNA; NC, negative control.

tein kinase kinase (MEK)-extracellular signal-regulated protein kinase (ERK) signaling pathway by PKC induces a specific pattern of gene expression that is required for the initiation of megakaryocytic differentiation (Racke et al., 2001; Elagib et al., 2003; Jacquel et al., 2006).

Small noncoding RNAs have recently been identified as regulators of gene expression. MicroRNAs (miRNAs) are a principal class of functional noncoding RNA molecules and play important roles in the regulation of gene expression (Bushati and Cohen, 2007). miRNAs regulate gene expression negatively by promoting mRNA degradation and/or inhibiting RNA translation through binding to a target site in the 3′ UTR of the target mRNA (Bartel, 2004; He and Hannon, 2004; Lim et al., 2005). miRNAs have diverse expression patterns and may regulate various physiological and disease processes (Ambros, 2004; Bartel, 2004; Plasterk, 2006; Tsuchiya et al., 2006, 2009). The role of miRNAs has been studied in various models of hematopoiesis (e.g., the down-regulation of miR-221 and miR-222 during erythroid differentiation) (Felli et al., 2005; Fabbri et al., 2008).

In the present study, we studied the biological function of miRNAs by using K562 cells as a model of megakaryocytic differentiation with a particular focus on the regulation of cell signaling. The expression profiling of miRNAs in these cells identified 21 miRNAs that were up-regulated during the PMA-induced megakaryocytic differentiation of K562 cells. Functional studies of three of the up-regulated miRNAs further revealed their roles during megakaryocytic differentiation: the miRNAs did not affect cell differentiation, rather they repressed cell proliferation by negatively regulating the MEK-ERK signaling cascade. Navarro et al. (2009) have recently reported similar experimental results with K562 cells as a model. We discuss where our study confirms their work and where our results extend or differ from theirs.

Materials and Methods

Materials. PMA was obtained from Sigma-Aldrich (St. Louis, MO). 1.4-diamino-2,3-dicyano-1,4-bis[2-amino-phenylthio]butadiene (U0126) was purchased from Promega (Madison, WI). Dimethyl sulfoxide (DMSO) was purchased from Nacalai Tesque (Kyoto, Japan). All other materials were from standard sources and of the highest purity that is available commercially.

Cell Culture and Staining. The human chronic myelocytic leukemia cell line K562 was cultured in RPMI 1640 medium (Invitrogen, Paisley, UK) supplemented with 10% heat-inactivated fetal bovine serum (Sigma-Aldrich) under 5% CO_2. The human embryonic kidney 293 cells were grown in 10% fetal bovine serum in Dulbecco's modified Eagle's medium (Sigma-Aldrich) at 37°C under 5% CO_2. K562 cells were fixed and stained with Giemsa stain (Wako Pure Chemicals, Tokyo, Japan) according to the manufacturer's instructions.

Reverse Transcription-Polymerase Chain Reaction Analysis. Total RNA was extracted from K562 cells by using ISOGEN (Nippon Gene, Tokyo, Japan) at 0, 3, 6, 12, 24, 48, 72, and 96 h after treatment with PMA (10 nM) or DMSO. RT-PCR analysis was performed as described previously (Ichimura et al., 2008). The sequences of the primers are shown in Supplemental Table 1.

Flow Cytometry Analysis. Cells (10^6) were incubated at 4°C for 30 min in 100 μl of phosphate-buffered saline that contained 0.1% bovine serum albumin and monoclonal antibodies against CD41-phycoerythrin or CD10-phycoerythrin (BD Biosciences, San Jose, CA) After three washes with phosphate-buffered saline, fluorescence was measured by using the FL2 channel of a FACSCalibur flow cytometer (BD Biosciences).

Expression Profiles of miRNAs in K562 Cells. The expression profiles of 253 miRNAs were determined by reverse transcription followed by quantitative real-time PCR (qRT-PCR) as described previously (Ruike et al., 2008). RNA input was normalized by using U6 snRNA as an endogenous control. Real-time PCR was performed by using a standard TaqMan PCR kit protocol on an ABI PRISM 7700 Sequence Detection System (Applied Biosystems, Foster City, CA). For data analysis, the threshold cycle (C_t) value was determined. The ΔC_t value was defined as the difference between the C_t value of a miRNA and the C_t value of U6. The normalized C_t values (ΔC_t) from PMA-treated cells were then compared with those from the corresponding vehicle-treated cells. The $\Delta\Delta C_t$ value was defined as the difference between the ΔC_t value from PMA-treated cells and the ΔC_t value from vehicle-treated cells.

Northern Blot Analysis. An aliquot of 10 μg of total RNA was separated by 15% denaturing polyacrylamide gel electrophoresis using the SequaGel Sequencing System (National Diagnostics, Atlanta, GA), transferred to a charged nylon membrane (PerkinElmer Life and Analytical Sciences, Waltham, MA) using a mini-trans-blot cell (Bio-Rad Japan, Tokyo, Japan). The RNA was cross-linked to the membrane and dried overnight. Aliquots of 20 pmol of the antisense oligonucleotides for miR-221, miR-222, and miR-34a were end-labeled with [γ^{32}P]dATP by using the MEGA-LABEL 5′-end labeling kit (Takara, Kyoto, Japan), and then purified by using a G-25 MicroSpin column (GE Healthcare, Little Chalfont, Buckinghamshire, UK). Prehybridization and hybridization were carried out by using ULTRAhyb-Oligo hybridization buffer (Ambion, Austin, TX) according to the manufacturer's protocol. The membrane was then exposed to autoradiography film. Subsequently, U6 small nuclear RNA (snRNA) was probed as a loading control, and the blot was exposed to autoradiography film for 5 min.

Western Blot Analysis. At 48 h after transfection, the cells were lysed in SDS sample buffer. Samples were separated by 10% SDS-polyacrylamide gels, transferred to polyvinylidene difluoride membranes, blocked for 1 h with 5% skim milk (Nacalai Tesque), and exposed to the primary antibody for 1 h. After incubation with a horseradish peroxidase-conjugated secondary antibody, immunoreactivity was detected by using the Western Lightning Chemiluminescence Reagent Plus kit (PerkinElmer Life and Analytical Sciences). Anti-p42/44 MAPK, anti-phospho-p42/44 MAPK, anti-MEK1, and anti-β-actin antibodies were used as the primary antibody. All primary antibodies used were purchased from Cell Signaling Technology (Danvers, MA) except anti-β-actin (Sigma-Aldrich).

Rapid Amplification of 5′ cDNA Ends. 5′RACE was carried out by using the 5′RACE System, version 2.0 (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. In brief, 5 μg of total RNA was reverse-transcribed by using the gene-specific primer GSP1 (5′-CCTGCATAGTAAGTGCTCTG-3′) and SuperScript II reverse transcriptase. A homopolymeric dC tail was then added to the 3′ end of the first-strand cDNA by using terminal deoxynucleotide transferase. The first round of PCR amplification was performed by using an abridged anchor primer and GSP2 (5′-CTACTATTCTC-CCTACGTGC-3′), and this was followed by nested PCR amplification using the Universal Amplification Primer and GSP3 (5′-CAGCTAAGACACTGCCAAAG-3′). Amplified PCR products were subcloned into pGEM-T Easy vector (Promega). The isolated clones were sequenced, and the BLAST program was used to search for corresponding genomic sequences.

Luciferase Reporter Analysis. Luciferase reporter assays were carried out with the Dual-Luciferase Reporter Assay System (Promega) as described previously (Raver-Shapira et al., 2007). For the promoter assays, approximately 10^5 K562 cells were plated in 24-well dishes and transfected with 720 ng of the reporter constructs and 80 ng of the pRL-TK vector (Promega), which encodes *Renilla reniformis* luciferase and provides an internal control, by using Lipofectamine 2000 Reagent (Invitrogen). Twenty-four hours after transfection, the cells were treated with PMA (10 nM) or vehicle

(1:10,000 DMSO) for 24 h and then luciferase activity was measured. A fragment of the hsa-miR-34a promoter, from −905 to +1 (P1), was amplified by PCR from human genomic DNA and subcloned into the pGL3-enhancer vector (Promega). A construct was also generated in which the AP-1 binding element (position −570) in the P1 fragment was mutated to a BamHI site. The p53-responsive distal promoter sequence in the miR-34a gene (P2) was cloned as described previously (Raver-Shapira et al., 2007). All constructs were confirmed by DNA sequencing.

For the luciferase reporter analysis, fragments of the 3′ UTRs that contained the target site/sites for miR-34a or miR-221/222 were amplified by PCR from genomic DNA and inserted into the XbaI site of the pGL3-promoter vector (Promega). Mutagenesis of the reporter plasmids was carried out with a QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). The sequences of the primers are shown in Supplemental Table 1. All constructs were confirmed by DNA sequencing. Human embryonic kidney 293 cells were cotransfected with 1 μg of the firefly luciferase reporter construct and 0.1 μg of the control vector pRL-Tk by using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. The cells were also transfected with the pre-miR-34a, pre-miR-221, or pre-miR-222 oli-

gonucleotides (PremiR miRNA Precursor Molecule; Applied Biosystems). Luciferase activities were measured 24 h after transfection as described previously (Terasawa et al., 2009).

Chromatin Immunoprecipitation Analysis. K562 cells were incubated in the presence or absence of PMA for 3 h. The cells were then cross-linked by adding formaldehyde, lysed, sonicated, and digested with micrococcal nuclease (New England Biolabs, Ispwich, MA). The resulting lysates were immunoprecipitated by using Dynabeads Protein G (Invitrogen) coupled with an antibody against c-Jun (Santa Cruz Biotechnology, Santa Cruz, CA), which is a component of the AP-1 transcription factor, or control IgG (Santa Cruz Biotechnology). The immunoprecipitated samples and an aliquot of the input lysate were digested with proteinase K and incubated at 65°C for 6 h to reverse the cross-links. The DNA fragments were purified by phenol/chloroform extraction and ethanol precipitation by using ethachinmate (Nippon Gene). The enrichment of DNA fragments that contained a consensus AP-1 binding site was assessed by real-time PCR (SYBR Premix ExTaq II; Takara) using the following primers: AP1BS-F, 5′-TTTCACGCAGACATGCTGAC-3′; AP1BS-R, 5′- CCTTCAGCAACTGTCTCTAC-3′.
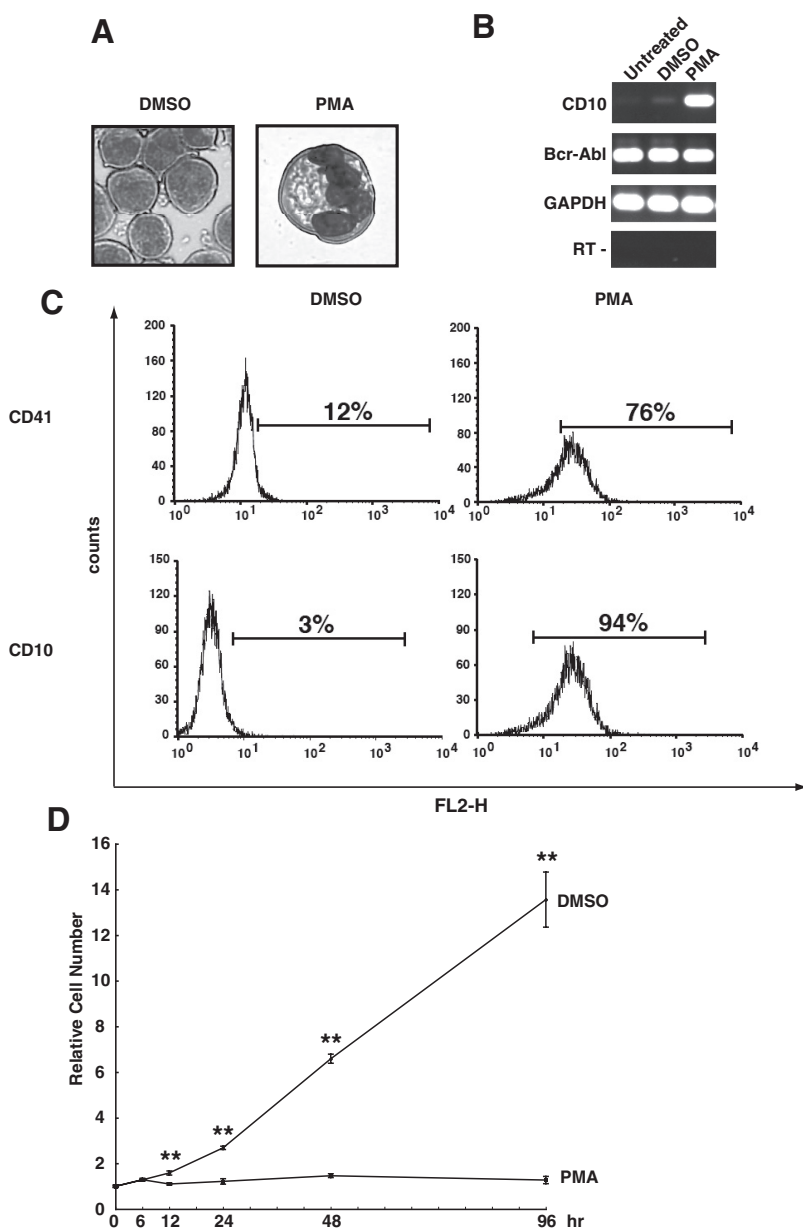

Fig. 1. PMA-induced differentiation of K562 cells. K562 cells were treated with 10 nM PMA for 96 h. A, morphology of differentiated K562 cells. Shown are preparations of K562 cells stained with Giemsa. B, RT-PCR analysis of CD10 expression. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and Bcr-Abl were used as invariant controls in the experiment. C, flow cytometry analysis of differentiation markers. A substantial increase in the binding of the anti-human CD10 (76%) and anti-human CD41 (94%) antibodies was observed in PMA-treated K562 cells at 96 h. D, cell numbers were counted at the indicated time points. The relative cell number at each time point shown on the growth curves represents the mean value ± S.E. of three independent experiments normalized to the cell number at the time of stimulation. ∗, $P < 0.05$ and ∗∗, $P < 0.01$ for PMA-treated cells versus vehicle-treated cells.

Transfection of Cells with miRNA Mimics and Inhibitors. Cells were seeded into six-well plates at a density of 1.25×10^5 cells per well 24 h before transfection. K562 cells were transfected by using the CodeBreaker siRNA Transfection Reagent (Promega) following the manufacturer's instructions. For each transfection, 300 pmol of precursor miRNA oligonucleotides (Applied Biosystems) in 250 μl of Opti-MEM (Invitrogen) was mixed with 250 μl of Opti-MEM that contained 10 μl of CodeBreaker reagent and had been preincubated for 15 min at room temperature. The mixture was incubated for 15 min at room temperature and then added to the cells. The cells were harvested 48 h after transfection for flow cytometry and Western blot analysis and reseeded for the analysis of cell proliferation.

Cells were also transfected with either a locked nucleic acid (LNA) probe against miRNA-34a (Exiqon, Vedbaek, Denmark) or a negative control probe (Exiqon) at a final concentration of 100 nM. LNA-modified anti-miRNA molecules interfere with miRNA function in a highly specific manner (Kaur et al., 2007). At 24 h after transfection, the cells were stimulated with PMA at a final concentration of 1 nM for the analysis of cell proliferation.

Cell Proliferation Assay. To measure cell proliferation, cells were plated in triplicate in 24-well plates and harvested. Cell num-bers were determined at various time points with a hemacytometer after dead cells had been excluded by staining with trypan blue.

Statistical Analysis. Unless otherwise indicated, the level of significance for the difference between data sets was assessed by using an unpaired Student's t test. Data are expressed as means $\pm$ S.E. $P < 0.05$ is considered to be statistically significant.

Results

Expression Profile of miRNAs during PMA-Induced Megakaryocytic Differentiation. Treatment with PMA (10 nM) for 96 h induced megakaryocytic differentiation in K562 cells as evidenced by morphological changes and enhanced expression of marker genes and surface antigens (Fig. 1, A–C). After 96 h of treatment, an increase in cell size and enhanced expression of CD41 and CD10, which were reported previously to be specific markers of megakaryocytic differentiation (Belhacène et al., 1998; Dorsey et al., 2002; Jacquel et al., 2006), were observed. Cell growth was arrested from 12 to 96 h after treatment with PMA.

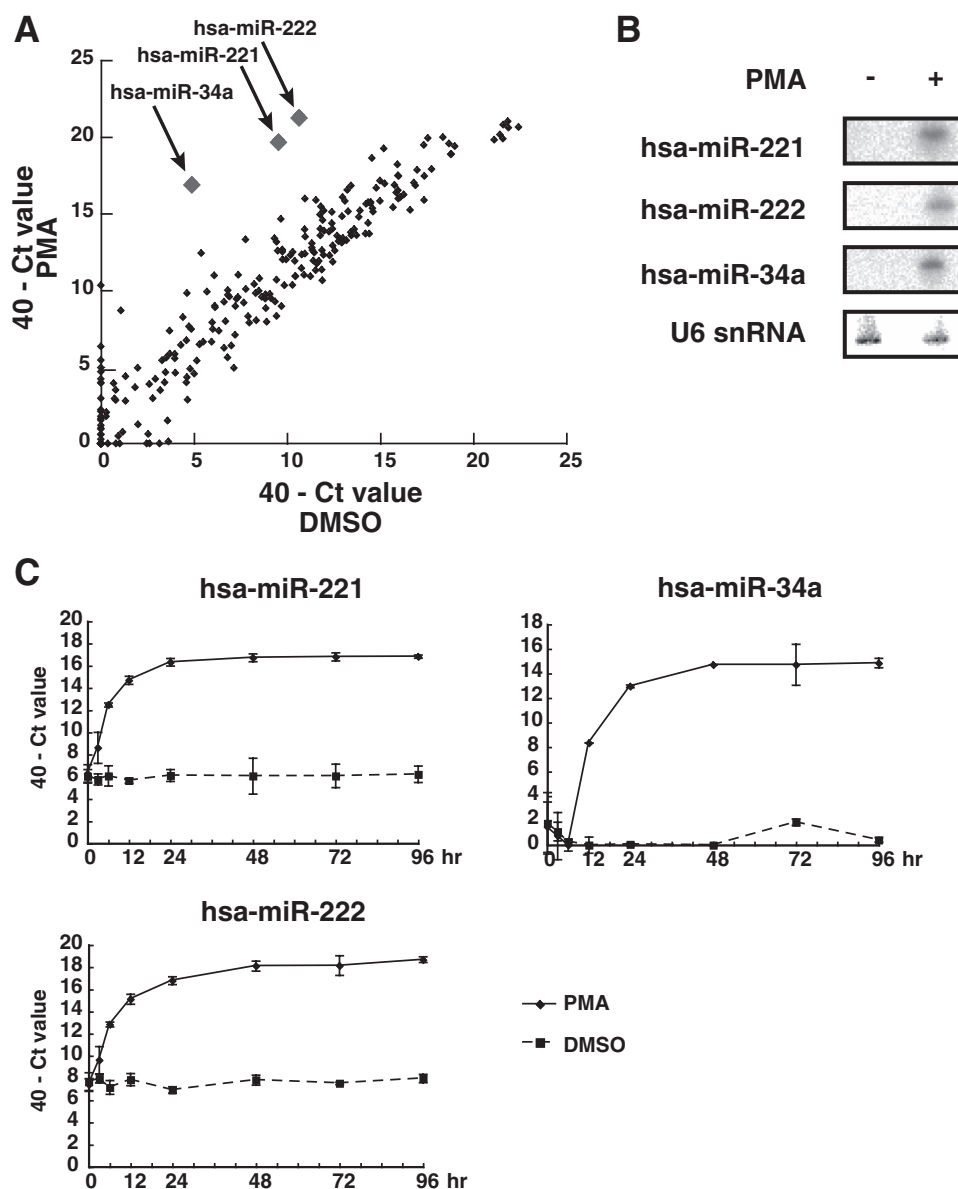To identify miRNAs that were expressed differentially during

Fig. 2. miRNA expression profiling of PMA-treated K562 cells by qRT-PCR and confirmation by Northern blotting. A, an aliquot of 5 ng of total RNA that had been extracted from K562 cells cultured with PMA (10 nM) or DMSO was analyzed by qRT-PCR. Shown is a graphic representation of the results. The data shown are the $40 - C_t$ values for each miRNA. B, Northern blotting was performed as described under *Materials and Methods* using probes with perfect complementarity to the mature sequences of miR-34a, miR-221, and miR-222. U6 snRNA was used as a loading control. C, time course of miRNA induction after PMA treatment. Taq-Man real-time PCR was performed at the indicated time points. The data shown are the $40 - C_t$ values for each miRNA. Bars represent the mean C_t values and corresponding S.E. for experiments performed in triplicate.

PMA-induced megakaryocytic differentiation, we first compared the miRNA expression profiles of PMA-treated cells with those of vehicle-treated cells after 96 h of treatment. Quantification of 253 different miRNAs by TaqMan real-time PCR showed that the expression of 21 miRNAs was up-regulated by PMA treatment (denoted by arrows in Fig. 2A). The up-regulated miRNAs, together with their predicted target genes and previously reported target genes, are summarized in Supplemental Table 2. Among these miRNAs, we further examined three miRNAs (miR-34a, miR-221, and miR-222), because the expression of those miRNAs was notably enhanced (~1000-fold) (Fig. 2A). Northern blotting confirmed the results of the qRT-PCR (Fig. 2B). A time course experiment after treatment with PMA showed that miR-34a, miR-221, and miR-222 expressed rapidly and persistently (Fig. 2C). Compared with the time course of expression of the miRNAs, the kinetics analysis showed that the activation of ERK occurred immediately (at least 5 min after PMA treatment) and was sustained to 12 h, when miR-34a and miR-221/222 were induced, but ERK was inactivated at 24 h after PMA treatment (Supplemental Fig. 1).

Induction of miRNAs via the MEK-ERK Signaling Pathway. Next, we performed cell signaling analysis to ascertain how PMA induced the expression of these miRNAs. The MEK-ERK pathway is associated with the induction of differentiation by PMA in K562 cells (Eriksson et al., 2005). Hence, we investigated whether the MEK-ERK pathway activated the transcription of miR-34a, miR-221, and miR-222. We blocked ERK activity by using the MEK1/2 inhibitor U0126. U0126 (20 μM) blocked the PMA-stimulated phosphorylation of ERK 1/2 (Fig. 3A) and inhibited the induction of miR-34a, miR-221, and miR-222 (Fig. 3B). The changes in cell morphology, growth arrest, and the expression of the marker genes were also inhibited by pretreatment with U0126 (Fig. 3C and data not shown).

To investigate the mechanism by which miR-34a was induced, we first determined the transcriptional start sites

of pre-miR-34a in PMA-treated K562 cells by 5'RACE analysis. The start sites of all the miR-34a transcripts were located within the proximal (~1 kilobase pair) promoter region (Fig. 4A). Luciferase reporter assays revealed the presence of PMA-inducible promoter activity within the proximal promoter region (Fig. 4B, P1), although the empty vector also had a low level of PMA-inducible activity (Fig. 4C). The mutation of the AP-1 binding element in the proximal promoter region significantly reduced the PMA-dependent activation of the promoter. The distal promoter region (Fig. 4B, P2) showed less promoter activity than the proximal region irrespective of whether it was exposed to PMA (Fig. 4, B and C). Furthermore, chromatin immunoprecipitation analysis using an AP-1-specific antibody revealed that PMA caused a 1.4-fold enrichment of the proximal promoter region of miR-34a in the immunoprecipitated DNA (Fig. 4D), whereas IgG resulted in no significant enrichment (data not shown).

Repression of MEK1 by miR-34a and c-*fos* by miR-221/222. Next, we performed a luciferase reporter assay to identify genes that were targeted by miR-34a or miR-221/222. We selected MEK1 as a candidate target of miR-34a and c-*fos* as a candidate target of miR-221/222 from ~300 genes that were predicted to be targets by the prediction algorithm TargetScan (Lewis et al., 2003). Given that MEK1 protein is significantly down-regulated after the treatment of K562 cells with PMA (Supplemental Fig. 2), we examined whether MEK1 was directly regulated by miR-34a. Transfection of the miR-34a precursor oligonucleotide significantly reduced luciferase expression from a reporter plasmid that contained the 3' UTR of MEK1 compared with the transfection of a scrambled RNA control (Fig. 5B). Likewise, the miR-221 or miR-222 precursor oligonucleotide reduced the activity of a reporter plasmid that contained the 3' UTR of c-*fos* (Fig. 5D). These RNA oligonucleotides had negligible effects on control plasmids.

Next, we examined the functional roles of these miRNAs. The ectopic expression of miR-221, miR-222, and miR-34a did
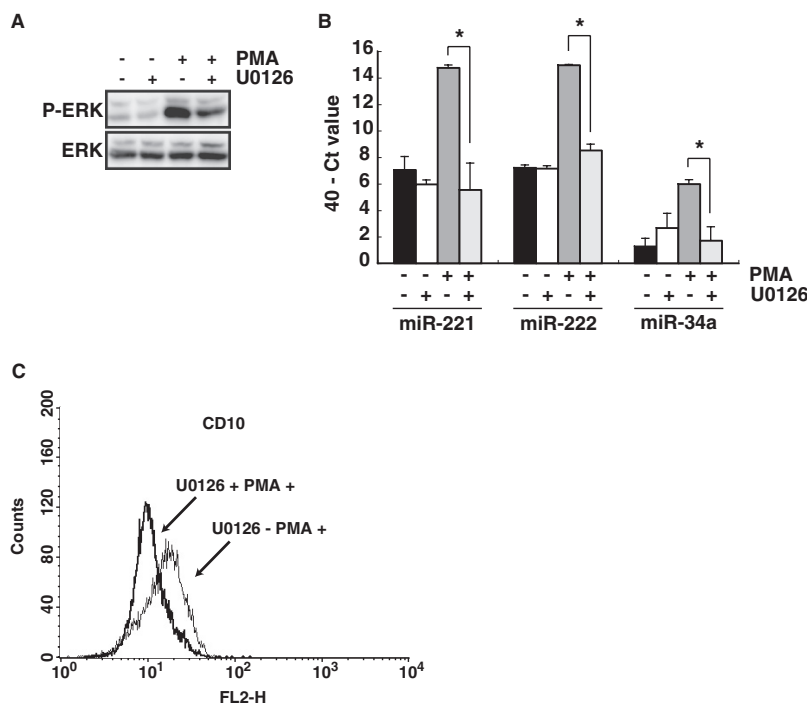
Fig. 3. Effect of U0126 on induction of the miRNAs. Cells were pretreated with U0126 (20 μM) for 0.5 h and then incubated with PMA (10 nM) for 12 h. A, the levels of ERK and phospho-ERK (P-ERK) in the cells were analyzed by Western blotting. B, the induction of miR-34a, miR-221, and miR-222 was analyzed by qRT-PCR. The data shown are the $40 - C_t$ values for each miRNA. Bars represent the mean C_t values and corresponding S.E. for experiments performed four times. ∗, $P < 0.05$ compared with the corresponding control. C, U0126 also inhibits differentiation. Flow cytometry analysis of differentiation markers was performed. Pretreatment with U0126 inhibits the expression of CD10.

not result in detectable changes in the expression of the marker genes (Fig. 6A) or cell morphology (data not shown), but cell proliferation was significantly reduced by the overexpression of miR-34a (Fig. 6B). Furthermore, Western blot analysis showed that miR-34a concomitantly reduced the level of MEK1 protein (Fig. 6C), whereas miR-221/222 did not affect cell numbers or the level of MEK1 protein (Fig. 6, B and C). PMA-induced activation of ERK was significantly decreased by overexpression of miR-34a (Fig. 6D). The MEK1 inhibitor U0126 inhibited ERK activation (data not shown) and repressed cell proliferation (Supplemental Fig. 3). Conversely, transfection of an LNA probe against miR-34a into PMA-treated K562 cells significantly ($P < 0.05$) enhanced both expression of MEK1 protein (~20%) and cell proliferation (Fig. 6E).

Discussion

In the present study, we examined the role of miRNAs in PMA-induced megakaryocytic differentiation by using K562
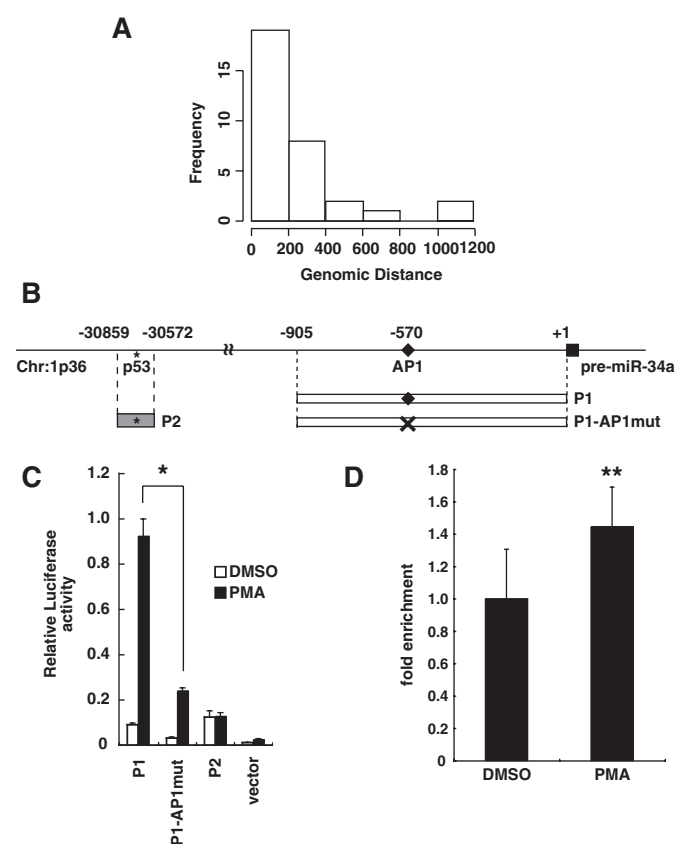

Fig. 4. Transactivation of miR-34a occurs in the proximal promoter region. A, 5′RACE was performed as described under *Materials and Methods*. The frequencies of the genomic distances are shown. The majority of transcripts were initiated in the proximal region. B, a schematic representation of the constructs. P2 indicates the position of the reported p53-responsive region. The asterisk shows the position of the p53-binding site. C, activity of promoter constructs in PMA or DMSO-treated K562 cells. Error bars represent the standard deviations from three independent transfections, each measured in triplicate. $P < 0.05$ for comparison indicated by ∗. D, chromatin immunoprecipitation analysis was carried out as described under *Materials and Methods*. The relative enrichment of DNA fragments that contained the consensus AP-1 binding site in the proximal promoter region of miR-34a was quantified by real-time PCR. The data represent the mean and corresponding S.E. of six experiments. ∗∗, $P < 0.01$ for PMA-treated cells versus vehicle (DMSO)-treated cells.

cells as a model. Expression profiling identified 21 miRNAs that were expressed differentially during differentiation. Among them, we focused on the miRNAs (miR-34a and miR-221/miR-222) whose expression was markedly altered during the megakaryocytic differentiation. Further studies showed that the activation of MEK-ERK signaling by PMA induced the expression of miR-34a, which then inhibited MEK1 ex-
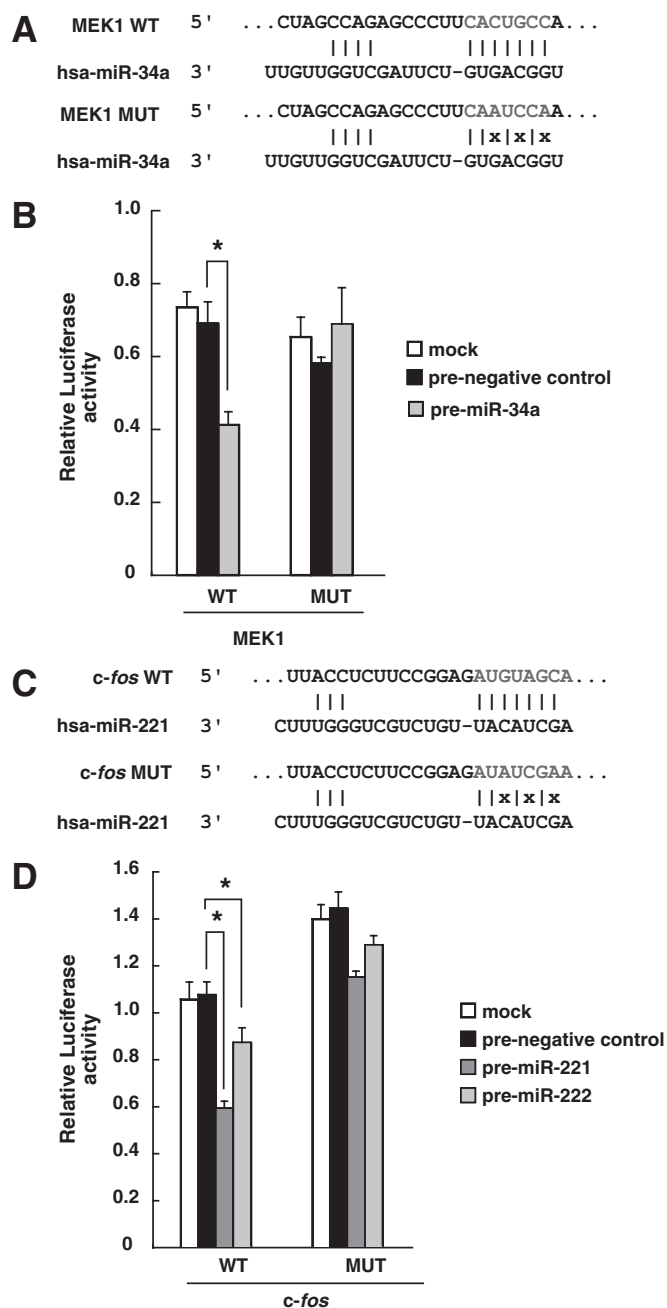

Fig. 5. Target validation for miR-34a, miR-221, and miR-222. Segments from the 3′ UTRs of genes that were predicted to contain the target site for miR-34a or miR-221 and miR-222 were cloned into the pGL3-promoter vector. A, top, miR-34a and the miR-34a-binding site in the 3′ UTR of MEK1. Bottom, miR-34a and the mutated miR-34a-binding site in the 3′UTR of MEK1. C, top, miR-221 and the miR-221/222-binding site in the 3′ UTR of c-*fos*. Bottom, miR-221 and the mutated miR-221/222-binding site in the 3′ UTR of c-*fos*. B and D, results are shown as the relative luciferase activity (the ratio of firefly luciferase/*R. reniformis* luciferase) for each combination of miRNA and reporter construct. The data represent the mean and corresponding S.E of three or four experiments. ∗, $P < 0.05$ compared with corresponding control.

pression, and led to the repression of cell proliferation. The results of this study revealed a potentially important role for miRNAs in the process of megakaryocytic differentiation.

In this study, we found that miR-34a was markedly in-

duced in K562 cells during PMA-induced megakaryocytic differentiation. Several groups have shown that p53 activates the transcription of miR-34a in HCT116 human colon cancer cells and H1299 human lung cancer cells (Chang et
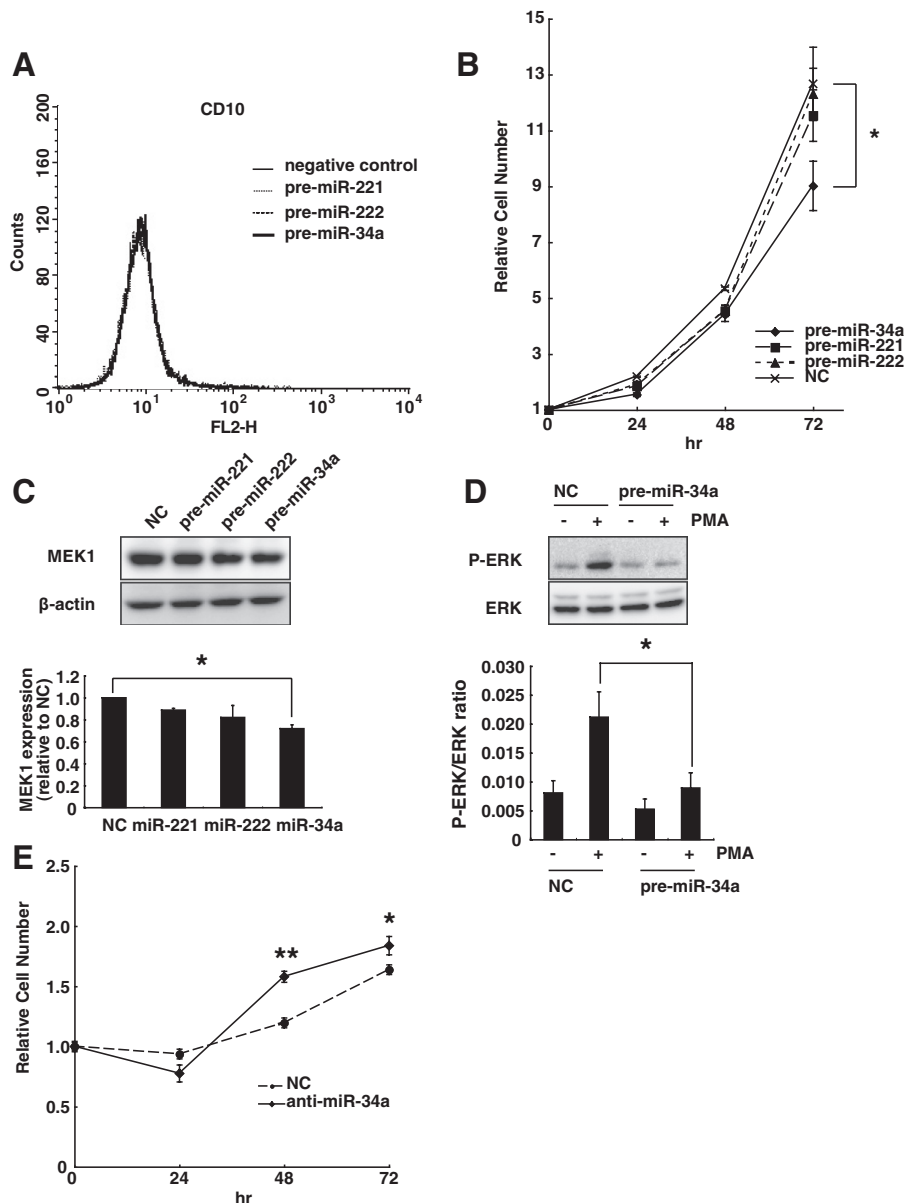

Fig. 6. A, flow cytometry analysis of differentiation markers. A detectable change in the binding of the anti-human CD10 antibodies was not observed. B, cell viability was assessed from days 2 to 5 after transfection of K562 cells with either pre-miR-34a, pre-miR-221, pre-miR-222, or a negative control (NC) oligonucleotide (100 nM) that does not code for any known miRNA. Cell populations that had been transfected with the NC oligo contained a significantly larger number of viable cells than those transfected with pre-miR-34a. Statistical differences between treatments were examined by one-way analysis of variance (ANOVA) with a Tukey-Kramer procedure for multiple comparisons. Differences were considered significant at $P < 0.05$. ∗, $P < 0.05$ for pre-miR-34a-transfected cells versus NC-transfected cells. C, K562 cells were transfected with pre-miR-34a, pre-miR-221, pre-miR-222, or a NC RNA (100 nM), and the levels of MEK1 and β-actin were analyzed by Western blotting (top). Representative results from one of three independent experiments are shown. Bottom, quantitative analysis of MEK1 protein normalized to β-actin levels in the respective samples. Values represent the mean and corresponding S.E. from three independent experiments. Statistical differences between treatments were examined by ANOVA with a Tukey-Kramer procedure for multiple comparisons. Differences were considered significant at $P < 0.05$. ∗, $P < 0.05$ for pre-miR-34a-transfected cells versus NC-transfected cells. D, K562 cells were transfected with pre-miR-34a or an NC RNA (100 nM). At 48 h after transfection, cells were treated with PMA (0.1 nM) for 5 min. Then, the levels of ERK and P-ERK were analyzed by Western blotting (top). Representative results from one of three independent experiments are shown. Bottom, quantitative analysis of P-ERK protein normalized to ERK levels in the respective samples. Values represent the mean and corresponding S.E. from three independent experiments. Statistical differences between treatments were examined by ANOVA with a Tukey-Kramer procedure for multiple comparisons. Differences were considered significant at $P < 0.05$. ∗, $P < 0.05$ for pre-miR-34a-transfected cells treated with PMA versus NC-transfected cells treated with PMA. E, at 1 day after transfection of an LNA probe against miR-34a (anti-miR-34a) or an NC probe (100 nM), cells were treated with PMA (1 nM). Cell viability was assessed from days 1 to 3 after PMA treatment. Cell populations that had been transfected with the probe against miR-34a contained a significantly larger number of viable cells than those transfected with the NC probe. Values represent the mean and corresponding S.E. from three independent experiments. ∗, $P < 0.05$ and ∗∗, $P < 0.01$ for anti-miR-34a-transfected cells versus NC-transfected cells.

al., 2007; Raver-Shapira et al., 2007). However, because K562 cells lack normal p53 function because of a single nucleotide insertion into the p53 coding sequence (Law et al., 1993), the induction of miR-34a expression during PMA-induced megakaryocytic differentiation of K562 cells was considered to occur independently of p53. The results of our study indicated that this induction of miR-34a expression by PMA can be regulated by MEK-ERK signaling. In addition, we identified a novel promoter region of the miR-34a that contained an AP-1 site, which was required for the maximal transactivation of miR-34a expression.

Using K562 cells as a model of megakaryocytic differentiation, Navarro et al. (2009) have recently performed a similar study to examine the potentially important role of miRNAs in this process. Our study confirmed some parts of their study, but our study differs and extends their study in other parts. Using miRNA microarray analysis, Navarro et al. (2009) identified 12 miRNAs that were up-regulated, whereas we identified 21 up-regulated miRNAs. Both studies found that the expression of miR-34a, miR-139, miR-375, miR-132/212, and miR-221/222 was significantly enhanced. Focusing on miR-34a, Navarro et al. (2009) observed that overexpression of miR-34a can induce megakaryocytic differentiation, concomitant with arrest of the cell cycle and proliferation; however, this effect required the presence of 0.1 nM PMA. Without PMA, we also observed that overexpression of miR-34a can lead to cell growth arrest, although the cells were not fully differentiated. Furthermore, we found that overexpression of miR-34a can inhibit the induction of ERK activation by PMA. In an effort to identify target genes of miR-34a in this process, Navarro et al. (2009) identified genes related to the cell cycle and cell differentiation (CDK4, CDK6, and MYB), the functions of which correlate well with the observed functions of miR-34a (arrest of cell cycle and cell proliferation). However, as Navarro et al. themselves admitted, the time courses for the expression of miR-34a and suppression of the target genes after PMA treatment were not well correlated; i.e., MYB and CDK4 were down-regulated before miR-34a was up-regulated. Unlike their study, our work identified MEK1 as a target of miR-34a and found that the expression of MEK1 showed an inverse correlation with the induction of miR-34a. By examining the transcriptional regulation of miR-34a, both studies observed that PMA induces the expression of miR-34a independently of p53, rather by activating an alternative phorbol ester-responsive promoter. However, the promoter sites identified by the two groups are different, and both sites are regulated by PMA stimulation. The promoter site we identified contained an AP-1 site, which prompted us to further study whether a pre-miR-34a transcript that was produced by this alternative phorbol ester-responsive promoter can negatively regulate ERK signaling by inhibiting MEK1. Our further studies indicated that this PMA-induced miR-34a could work as a "negative feedback" mechanism, which highlights a novel function of miRNA. In summary, using the same model of cell differentiation, we confirmed the study of Navarro et al. (2009) with respect to the expression of miRNAs and their functional roles during megakaryocytic differentiation. Furthermore, our study extended the previous study by demonstrating that the MEK-ERK pathway, after activation by PMA, can induce

the expression of miRNAs that in turn negatively regulate the MEK-ERK signaling pathway.

The MEK-ERK–c-Fos pathway, which is activated during PMA-induced megakaryocytic differentiation, seems to be negatively regulated by miRNAs at multiple points. Of the up-regulated miRNAs that we identified, we demonstrated that miR-34a and miR-221/222 targeted MEK1 and c-*fos*, respectively. Although the transfection of none of the three miRNAs led to megakaryocytic differentiation (as shown by the lack of expression of megakaryocytic marker genes or morphological changes), transfection of miR-34a caused cell growth arrest, which is one of the significant events that occur during the PMA-induced differentiation of K562 cells. In addition, owing to the fact that the activation of AP-1 by ERK is required for the megakaryocytic differentiation of K562 cells (Eriksson et al., 2005), and because c-Fos is a component of the AP-1 transcription factor complex, repression of c-Fos by miR-221/miR-222 can inhibit the megakaryocytic differentiation. Furthermore, miR-221/miR-222 were shown to suppress the expression of c-kit, which is required for the erythroid differentiation of hematopoietic precursor cells, including K562 cells (Felli et al., 2005). Taken together, the results of our study might indicate that the activation of the MEK-ERK–c-Fos pathway is required for the PMA-induced megakaryocytic differentiation, and that it also turns on a mechanism of negative feedback for the signaling pathway by inducing the expression of miRNAs. PMA-induced activation of ERK can be controlled by multiple mechanisms [e.g., PMA-induced down-regulation of PKC and the guanine nucleotide exchange factor RasGRP (Ehrhardt et al., 2004)], and there are various negative feedback mechanisms that act on ERK activation, for example, involving MAPK phosphatases (Owens and Keyse, 2007). The results of our study demonstrated that miR-34a suppressed the expression of MEK1 at the post-transcriptional level, leading to the repression of PMA-dependent ERK activation. Hence, miR-34a may also have an important role in MEK-ERK signaling and this regulation might be one mechanism by which the differentiated state of the cell can be fine-tuned. In conclusion, megakaryocytic differentiation seems to be a complex process that involves multiple molecular events, and our present study highlights a potentially important role for miRNAs in the regulation of signal transduction mechanisms in this process.

References

Ambros V (2004) The functions of animal microRNAs. *Nature* **431:**350–355.
Bartel DP (2004) MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell* **116:**281–297.
Belhacène N, Maulon L, Guérin S, Ricci JE, Mari B, Colin Y, Cartron JP, and Auberger P (1998) Differential expression of the Kell blood group and CD10 antigens: two related membrane metallopeptidases during differentiation of K562 cells by phorbol ester and hemin. *FASEB J* **12:**531–539.
Bushati N and Cohen SM (2007) MicroRNA functions. *Annu Rev Cell Dev Biol* **23:**175–205.
Chang TC, Wentzel EA, Kent OA, Ramachandran K, Mullendore M, Lee KH, Feldmann G, Yamakuchi M, Ferlito M, Lowenstein CJ, et al. (2007) Transactivation of miR-34a by p53 broadly influences gene expression and promotes apoptosis. *Mol Cell* **26:**745–752.
Dorsey JF, Cunnick JM, Mane SM, and Wu J (2002) Regulation of the Erk2–Elk1 signaling pathway and megakaryocytic differentiation of Bcr-Abl(+) K562 leukemic cells by Gab2. *Blood* **99:**1388–1397.
Ehrhardt A, David MD, Ehrhardt GR, and Schrader JW (2004) Distinct mechanisms determine the patterns of differential activation of H-Ras, N-Ras, K-Ras 4B, and M-Ras by receptors for growth factors or antigen. *Mol Cell Biol* **24:**6311–6323.
Elagib KE, Racke FK, Mogass M, Khetawat R, Delehanty LL, and Goldfarb AN (2003) RUNX1 and GATA-1 coexpression and cooperation in megakaryocytic differentiation. *Blood* **101:**4333–4341.
Eriksson M, Arminen L, Karjalainen-Lindsberg ML, and Leppä S (2005) AP-1

regulates α2β1 integrin expression by ERK-dependent signals during megakaryocytic differentiation of K562 cells. *Exp Cell Res* **304:**175–186.

Fabbri M, Garzon R, Andreeff M, Kantarjian HM, Garcia-Manero G, and Calin GA (2008) MicroRNAs and noncoding RNAs in hematological malignancies: molecular, clinical and therapeutic implications. *Leukemia* **22:**1095–1105.

Felli N, Fontana L, Pelosi E, Botta R, Bonci D, Facchiano F, Liuzzi F, Lulli V, Morsilli O, Santoro S, et al. (2005) MicroRNAs 221 and 222 inhibit normal erythropoiesis and erythroleukemic cell growth via kit receptor down-modulation. *Proc Natl Acad Sci USA* **102:**18081–18086.

He L and Hannon GJ (2004) MicroRNAs: small RNAs with a big role in gene regulation. *Nat Rev Genet* **5:**522–531.

Ichimura A, Kadowaki T, Narukawa K, Togiya K, Hirasawa A, and Tsujimoto G (2008) In silico approach to identify the expression of the undiscovered molecules from microarray public database: identification of odorant receptors expressed in non-olfactory tissues. *Naunyn Schmiedebergs Arch Pharmacol* **377:**159–165.

Ishiko E, Matsumura I, Ezoe S, Gale K, Ishiko J, Satoh Y, Tanaka H, Shibayama H, Mizuki M, Era T, et al. (2005) Notch signals inhibit the development of erythroid/megakaryocytic cells by suppressing GATA-1 activity through the induction of HES1. *J Biol Chem* **280:**4929–4939.

Jacquel A, Herrant M, Defamie V, Belhacene N, Colosetti P, Marchetti S, Legros L, Deckert M, Mari B, Cassuto JP, et al. (2006) A survey of the signaling pathways involved in megakaryocytic differentiation of the human K562 leukemia cell line by molecular and c-DNA array analysis. *Oncogene* **25:**781–794.

Kaur H, Babu BR, and Maiti S (2007) Perspectives on chemistry and therapeutic applications of locked nucleic acid (LNA). *Chem Rev* **107:**4672–4697.

Kim KW, Kim SH, Lee EY, Kim ND, Kang HS, Kim HD, Chung BS, and Kang CD (2001) Extracellular signal-regulated kinase/90-KDA ribosomal S6 kinase/nuclear factor-κB pathway mediates phorbol 12-myristate 13-acetate-induced megakaryocytic differentiation of K562 cells. *J Biol Chem* **276:**13186–13191.

Lam LT, Ronchini C, Norton J, Capobianco AJ, and Bresnick EH (2000) Suppression of erythroid but not megakaryocytic differentiation of human K562 erythroleukemic cells by notch-1. *J Biol Chem* **275:**19676–19684.

Law JC, Ritke MK, Yalowich JC, Leder GH, and Ferrell RE (1993) Mutational inactivation of the p53 gene in the human erythroid leukemic K562 cell line. *Leuk Res* **17:**1045–1050.

Lewis BP, Shih IH, Jones-Rhoades MW, Bartel DP, and Burge CB (2003) Prediction of mammalian microRNA targets. *Cell* **115:**787–798.

Lim LP, Lau NC, Garrett-Engele P, Grimson A, Schelter JM, Castle J, Bartel DP, Linsley PS, and Johnson JM (2005) Microarray analysis shows that some microRNAs down-regulate large numbers of target mRNAs. *Nature* **433:**769–773.

Navarro F, Gutman D, Meire E, Cáceres M, Rigoutsos I, Bentwich Z, and Lieberman J (2009) miR-34a contributes to megakaryocytic differentiation of K562 cells independently of p53. *Blood* **114:**2181–2192.

Owens DM and Keyse SM (2007) Differential regulation of MAP kinase signalling by dual-specificity protein phosphatases. *Oncogene* **26:**3203–3213.

Pettiford SM and Herbst R (2003) The protein tyrosine phosphatase HePTP regulates nuclear translocation of ERK2 and can modulate megakaryocytic differentiation of K562 cells. *Leukemia* **17:**366–378.

Plasterk RH (2006) Micro RNAs in animal development. *Cell* **124:**877–881.

Racke FK, Wang D, Zaidi Z, Kelley J, Visvader J, Soh JW, and Goldfarb AN (2001) A potential role for protein kinase C-ε in regulating megakaryocytic lineage commitment. *J Biol Chem* **276:**522–528.

Raver-Shapira N, Marciano E, Meiri E, Spector Y, Rosenfeld N, Moskovits N, Bentwich Z, and Oren M (2007) Transcriptional activation of miR-34a contributes to p53-mediated apoptosis. *Mol Cell* **26:**731–743.

Ruike Y, Ichimura A, Tsuchiya S, Shimizu K, Kunimoto R, Okuno Y, and Tsujimoto G (2008) Global correlation analysis for micro-RNA and mRNA expression profiles in human cell lines. *J Hum Genet* **53:**515–523.

Shelly C, Petruzzelli L, and Herrera R (1998) PMA-induced phenotypic changes in K562 cells: MAPK-dependent and -independent events. *Leukemia* **12:**1951–1961.

Terasawa K, Ichimura A, Sato F, Shimizu K, and Tsujimoto G (2009) Sustained activation of ERK1/2 by NGF induces microRNA-221 and 222 in PC12 cells. *FEBS J* **276:**3269–3276.

Tsuchiya S, Oku M, Imanaka Y, Kunimoto R, Okuno Y, Terasawa K, Sato F, Tsujimoto G, and Shimizu K (2009) MicroRNA-338–3p and microRNA-451 contribute to the formation of basolateral polarity in epithelial cells. *Nucleic Acids Res* **37:**3821–3827.

Tsuchiya S, Okuno Y, and Tsujimoto G (2006) MicroRNA: biogenetic and functional mechanisms and involvements in cell differentiation and cancer. *J Pharmacol Sci* **101:**267–270.

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