Novel functions for mda-7/IL-24 and IL-24 delE5: regulation of differentiation of acute myeloid leukemic cells

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Abstract

Characterizing genes associated with leukemic cells differentiation may provide help for understanding mechanisms on the leukemia differentiation. The aim of this study is to investigate whether the expression of melanoma differentiation associated gene-7/interleukin-24 (mda-7/IL-24) could be induced during leukemia differentiation, and whether mda-7/IL-24 plays a role in leukemia differentiation. We showed that the expression of mda-7/IL-24 and IL-24 delE5, a mda-7/IL-24 splice variant, was induced in U937 and HL60 cells during 12-O-tetradecanoylphorbol-13-acetate (TPA)-mediated monocytic differentiation. Activation of MAPK/ERK pathway was required for their induction. Knockdown of mda-7/IL-24 and IL-24 delE5 resulted in significant inhibition of the monocytic differentiation induced by TPA. More importantly, ectopic overexpression of mda-7/IL-24 and IL-24 delE5 significantly induced U937, HL60 cells, and blast cells from patients with acute myeloid leukemia (AML)–M5 to differentiate, while normal hematopoietic progenitors were not affected. Furthermore, the molecular effector associated with selective differentiation induction by mda-7/IL-24 and IL-24 delE5 may be reactive oxygen species (ROS), and the source of ROS generation was nicotinamide adenine dinucleotide phosphate (NADPH) oxidase. Taken together, our results reveal the mechanism by which TPA induces monocytic differentiation, and show for the first time the specific differentiation-inducing effects of mda-7/IL-24 and IL-24 delE5 on human myeloid leukemic cells.

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Introduction

Acute myeloid leukemia (AML) is characterized by a block in myeloid differentiation.

Currently, most tumor treatment strategies are mainly focused on killing cancerous cells

using chemotherapy, which has a number of toxic side effects and other important

disadvantages. An alternate approach for treating cancer employs agents that modify

tumor growth by inducing terminal differentiation, a process termed "differentiation

therapy ". Since the establishment of all-trans retinoic acid (ATRA) as a differentiation

therapy to treat acute promyelocytic leukemia (APL), APL has become a curable subtype

of acute myelocytic leukemia (1). Extensive efforts are being directed towards

characterizing genes associated with leukemic cells differentiation. Achieving this goal

may provide help for understanding mechanisms on the AML cells differentiation and

designing safe and specific differentiation therapy to treat leukemia.

Melanoma differentiation-associated gene-7 (mda-7) was first identified as a gene

whose expression correlates with the induction of terminal differentiation in human

melanoma cells. Based on its chromosomal location, sequence homology, structure, and

cytokine-like properties, mda-7 has also been named interleukin-24 (IL-24) (2). Two

splice variants of mda-7/IL-24 have been reported. One lacks exon 5 (IL-24 delE5), and

the other lacks both exon 3 and exon 5 (mda-7s) (3). mda-7s has minimal sequence

homology to mda-7/IL-24 but functionally interacts with and inhibits the secretion of the

wild-type protein (4). In contrast, the IL-24 delE5 splice variant has high homology to

mda-7/IL-24, but lacks the IL-10 signature motif and is weakly amplified in melanocytes

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(3). The function of IL-24 delE5 remains unknown.

Many studies have focused on the apoptosis inducing effect of mda-7/IL-24 on a broad spectrum of human cancers (2, 5). The protein product of mda-7/IL-24 can exert its antitumor effect by binding to its two receptor complexes, IL-20R1/IL-20R2 or IL-22R1/IL-20R2 (6, 7). Interestingly, when introduced into tumor cells by adenovirus transduction (Ad. mda-7), mda-7/IL-24 has a potent intracellular mode of action and is active through a receptor-independent mechanism (2, 6, 8). Although mda-7/IL-24 expression correlates with induction of terminal differentiation in human melanoma cells, ectopic overexpression of mda-7/IL-24 causes growth suppression and apoptosis but no induction of differentiation in melanoma cells (9).

12-O-tetradecanoylphorbol-13-acetate (TPA) is a well-known agent that signals differentiation of myeloid precursors and myeloid leukemia cell lines (10, 11). It has been reported that TPA could increase the mda-7/IL-24 promoter activity in melanoma cells and HeLa cells (12, 13). These data brings up an interesting possibility that mda-7/IL-24 can be induced during TPA-mediated leukemia differentiation. Herein we find the expression of mda-7/IL-24 is induced when TPA induces U937 and HL60 cells to undergo monocytic differentiation. Intriguingly, the expression of IL-24 delE5, a mda-7/IL-24 splice variant, is also induced during this process. Furthermore, we demonstrate that mda-7/IL-24 and IL-24 delE5 play an important role in the monocytic differentiation in leukemia cells mediated by TPA, and ectopic expression of mda-7/IL-24 and IL-24 delE5 induced myeloid leukemic cells to differentiate, without affecting normal hematopoietic

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progenitors. The molecular effector associated with their selective differentiation

induction effect may be reactive oxygen species (ROS).

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Materials and Methods

Cell lines and reagents

HL60 (promyelocytic leukemia) and U937 (promonocytic leukemia) used in the research were obtained directly from American Type Culture Collection (ATCC) and were cultured as described (14). These cell lines were passaged in our laboratory for fewer than 6 months after receipt. ATCC characterized these cell lines by DNA profiling.

TPA (Sigma, St Louis, MO, USA) was added to cells at a final concentration of 20 nM for U937 and HL60 cells. The mitogen activated protein kinase kinase (MEK) inhibitors U0126 and PD98059, and JNK inhibitor SP600125 were purchased from Calbiochem (San Diego, CA, USA). N-acetylcysteine (NAC) and diphenyleneiodonium (DPI) were purchased from Sigma.

RT-PCR

cDNA was synthesized by reverse transcribed of 2 μg RNA using a commercial kit (Promega Corporation, Madison, WI, USA). The following primers was used to amplify cDNA (2 μl) (forward/reverse): mda-7/IL-24 and IL-24 delE5: 5′-ATGAATTTTCA ACAGAGGC-3′/5′-TCAGAGCTTGTAGAATTT-3′; GAPDH: 5′-TGAAGGTCGGAGTCAAC GGATTTGG-3′/5′-CATGTGGGCCATGAGGTCCACCAC-3′. The products were sequenced using an ABI PRISM 3770 apparatus (Perkin Elmer Corp., Foster City, CA, USA).

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Real-time RT–PCR

Real-time quantitative RT-PCR was performed using the SYBR Premix Ex Taq^{TM} kit

(Perfect Real Time; TaKaRa Biotechnology, Dalian, China) and Applied Biosystems 7500

Sequence Detector System, according to the manufacturer's instructions. Primers

specific to mda-7/IL-24 and IL-24 delE5 were used as follows (forward/reverse):

mda-7/IL-24: 5'-AAGTAGAACTCCAGCAGGGTG-3'/5'-TGGGCTGTGAAAGACACTA-3';

IL-24 delE5: 5'-CATTTTCTTGGCTCTCAGCATC-3'/5'-TGGGCTGTGAAAGACACTA-3'.

The comparative threshold cycle method was used for calculation of fold-amplification.

The expression level of each gene was normalized to the expression level of GAPDH.

RNA interference

mda-7/IL-24 siRNA (100 nM; Dharmacon, Chicago, IL., USA. Cat # LQ-007977-00) and

non-targeting siRNA were transfected into cells using nucleofection (Amaxa, GmbH, Köln,

Germany).

Plasmid Construction and transfection

The cDNAs for mda-7/IL-24 or IL-24 delE5 were cloned into the plasmid pIRES (BD

Bioscience Clontech, Palo Alto, CA, USA) using the same primers (forward/reverse):

5′-ATTGGATCCGGAACACGAGACTGAGAGATG-3′/5′-ATGTCGACAGGGAACAAACCA

GTGCCA-3′, and termed IRES-mda-7/IL-24 and IRES-IL-24 delE5, respectively. The

resulting constructs were purified with a QIAGEN EndoFree Plasmid kit (QIAGEN, Hilden,

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Germany) and verified by sequencing.

Transient transfection was performed by using Amaxa cell optimization kit V (Amaxa GmbH). Cells were nucleofected using program W-001 for U937, T-019 for HL60, S-004 for primary bone marrow mononuclear cells (BMMCs), and U-001 for CD34^{+} cells.

Proliferation assay, colony forming assay, animal xenograft tumor model and apoptosis analysis.

See Supplementary Materials and Methods for details.

FACS assay for in vitro differentiation

The expression of differentiation-specific cell surface antigens was measured using CD11b-PE, CD14-FITC, and CD115-PE monoclonal antibodies (BD Pharmingen, San Diego, CA, USA) by flow cytometry. The isotype-matched control antibodies IgG1-PE and IgG1-FITC were used to measure background fluorescence.

Western blot analysis

Western blot analysis was performed using standard techniques. Proteins were detected with primary antibodies against mda-7/IL-24 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), phosphorylated ERK1/2 or ERK1/2, phosphorylated JNK or JNK, phosphorylated p38 MAPK or p38 MAPK (Cell Signaling Technology Inc, Danvers, MA, USA) or β-actin (Santa Cruz Biotechnology).

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Patient samples and cord blood mononuclear cell isolation and culture

Bone marrow (BM) samples from patients with AML (French-American-British [FAB] classification M5) were obtained at our hospital. The percentage of blasts in the BM was over 70% for all samples. BMMCs were isolated and maintained in RPMI 1640 medium with 10% FCS at 37°C.

Cord blood was obtained from healthy post partum women. Mononuclear cells (MNCs) were separated, and CD34$^+$ cells were purified using the MiniMacs separation system (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's recommendations. The purity of isolated CD34$^+$ cells was greater than 95% as assessed with FACS using the anti-CD34-PE monoclonal antibody (BD Pharmingen).

Purified CD34$^+$ cells were transfected with IRES-mda-7/IL-24 or IRES-IL-24 delE5, and then cultured in liquid medium and methylcellulose medium. For liquid culture, 1×10^5 cells/ml were seeded into 24-well culture plates in Stemline^{TM} II Hematopoietic Stem Cell Expansion Medium (Sigma) containing 100 ng/ml SCF, 50 ng/ml TPO, and 100 ng/ml Flt-3 (Peprotech, Offenbach, Germany). For methylcellulose culture, 5×10^3 cells were suspended in 1 ml methylcellulose media (MethoCult H4434, Stem cell technologies INC, Palo Alto, CA, USA) and then incubated at 37°C for 14 days. The granulocyte-macrophage colony-forming unit (CFU-GM) colonies, erythroid burst-forming unit (BFU-E) colonies, and granulocyte-erythrocyte-monocyte-macrophage colony-forming unit (CFU-GEMM) colonies that consisted of more than 50 cells were

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scored. Colonies were confirmed by cytologic analysis after removal from the dish by micropipette and staining with Wright-Giemsa staining.

Ethical clearance was obtained from the Institute Ethics Committee and all participants gave informed consent.

FACS assay for ROS production

Cells were incubated with 10 µM 2′, 7′-dichlorodihydrofluorescein diacetate (H2DCF-DA) (Beyotime Institute of Biotechnology, Haimen, China) in PBS for 20 min at 37°C in the dark. The fluorescence intensity was monitored with an excitation wavelength of 488 nm and an emission wavelength of 530 nm.

Measurement of NADPH oxidase activity

NADPH oxidase activity was analyzed as described (15). For analysis of specific oxidase activity, the rate of consumption of NADPH inhibited by DPI was measured, by adding 10 µM DPI (sigma) 30 min before the assays. All measurements were expressed in nanomoles of substrate/min/10^6 cells. Control cultures were treated with the solvent DMSO.

Statistical analysis

Statistical analysis was done with ANOVA, *t*-test, and χ^2 using the SPSS software package (SPSS, Chicago, IL, USA). Differences were considered to be statistically

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significant when p < 0.05. All results are representative of at least three individual

experiments.

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Results

TPA induces the expression of mda-7/IL-24 and IL-24 delE5 in U937 and HL60 cell lines

We evaluated whether mda-7/IL-24 expression could be induced by TPA in U937 and HL60 cells. The expression of mda-7/IL-24 mRNA was first detected using RT-PCR with primers designed to amplify the full-length mda-7/IL-24 open reading frame, which facilitated detection of variant transcripts. We observed that mda-7/IL-24 mRNA expression could not be detected in the untreated cell lines, but was induced at 48 hours after TPA treatment (Fig. 1A). Interestingly, in addition to the full-length mda-7/IL-24 transcript, an additional smaller transcript was also detected in U937 and HL60 cells during TPA-induced monocytic differentiation. Isolation, cloning, and sequencing of this transcript showed that the smaller species was a mda-7/IL-24 splice variant IL-24 delE5, which lacks exon 5 of the full-length mda-7/IL-24.

We then used real-time PCR to quantitate the expression of mda-7/IL-24 and IL-24 delE5, and found that the induction of both mda-7/IL-24 and IL-24 delE5 began to increase at 24 hours after TPA treatment (Supplementary Fig. S1A). Dose-response study showed that mda-7/IL-24 and IL-24 delE5 was induced dose dependently when U937 and HL60 cells were treated with TPA at concentrations less than 20 nM (data not shown). The relative expression levels of IL-24 delE5 and mda-7/IL-24 were also tested using quantitative real-time RT-PCR. The results showed that IL-24 delE5 mRNA levels were 20% of the levels of mda-7/IL-24 mRNA in U937 cells, and 30% of the levels of

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mda-7/IL-24 mRNA in HL60 cells (Fig. 1B). The specificity of the mda-7/IL-24 and IL-24 delE5 primer sets was confirmed by sequencing PCR products and melting curve analysis. The mda-7/IL-24 and IL-24 delE5 proteins were analyzed by western blotting with a mda-7/IL-24-specific antibody recognizing regions common to both mda-7/IL-24 and IL-24 delE5. The two proteins were approximately 35 kDa and 24 kDa, which are consistent with the predicted molecular weights of mda-7/IL-24 and IL-24 delE5, respectively (Fig. 1C).

The monocytic differentiation of U937 and HL60 cells induced by TPA were monitored by detecting the differentiation-specific cell surface antigens CD11b, CD14 and CD115 using FACS assay. We found that these markers began to increase at 24 hours after TPA treatment and reached a maximal level at 72 hours (Supplementary Fig. S1B).

We also detected the induction of other members of the IL-10 family of cytokines, IL-19, IL-20, IL-22, and IL-26 by TPA. Our results showed that mda-7/IL-24 is the only cytokine consistently upregulated in both U937 and HL60 cells (data not shown), suggesting that mda-7/IL-24 is the specific cytokine of IL-10 family induced by TPA in the two cell lines.

mda-7/IL-24 and IL-24 delE5 play an important role in TPA-induced differentiation of U937 and HL60 cells

Our current study showed that expression of mda-7/IL-24 and IL-24 delE5 was induced when TPA induced U937 and HL60 cells to undergo monocytic differentiation. A key

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question is whether their expression is required for TPA-mediated differentiation. To test

this hypothesis, siRNA corresponding to mda-7/IL-24 and IL-24 delE5 was transfected into

U937 and HL60 cells using nucleofection before addition of TPA. U937 and HL60 cells

transduced with siRNA resulted in a 60% to 70% reduction of mda-7/IL-24 and IL-24 delE5

protein expression in more than 90% of the transfected cells, compared with the

non-targeting control (Fig. 2A). Inhibition of mda-7/IL-24 and IL-24 delE5 expression

resulted in a significant decrease in the level of differentiated monocyte/macrophage

surface markers CD11b, CD14, and CD115 ($p < 0.05$, Fig. 2B), and fewer changes in cell

morphology induced by TPA (Supplementary Fig. S2). These data indicate that the

reduction of TPA-induced differentiation is associated with mda-7/IL-24 and IL-24 delE5

inhibition.

Involvement of the MAPK/ERK signaling pathway in mda-7/IL-24 and IL-24 delE5

expression induced by TPA in U937 and HL60 cells

TPA activates PKC, and it has been reported that PKC can activate extracellular

signal-regulated kinases (ERKs) (16). To further elucidate the mechanism by which TPA

induces the expression of mda-7/IL-24 and IL-24 delE5, we measured the effects of TPA

on activation of ERK1/2. As shown in Fig. 3A, treatment with TPA for 12 hours induced a

significant increase in phospho-ERK1/2 in U937 and HL60 cells compared with untreated

cells. This phosphorylation level began to decrease after 24 hours and returned to the

normal level at 48 hours. Stripping and subsequent reprobing of the same blot with an

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antibody against total ERK1/2 demonstrated no significant change in total ERK1/2 expression, indicating that treatment with TPA induces the activation of pre-existing ERK1/2.

Then, two MEK inhibitors, U0126 and PD98059, were used to demonstrate whether the phosphorylation of ERK1/2 is a phenomenon that regulates the expression of mda-7/IL-24 and IL-24 delE5 and the differentiation induced by TPA. As shown in Fig. 3B, the expression of mda-7/IL-24 and IL-24 delE5 induced by TPA was significantly attenuated by the two inhibitors. Moreover, as shown in Fig. 3C and D, adherence and differentiation of TPA-treated U937 and HL60 cells were almost completely inhibited by pretreatment with U0126 (10 μM) and partially inhibited by PD98059 (25 μM) ($P < 0.001$), whereas cell viability and proliferation were not significantly affected by the two inhibitors (data not shown).

We also evaluated the roles of the other two kinds of MAPK, c-Jun N-terminal kinases (JNK) and p38 mitogen-activated protein kinases (p38 MAPK), and showed that TPA treatment could not activate p38 MAPK, and although TPA significantly increased the phosphorylation level of JNK, the JNK inhibitor SP600125 did not inhibit induction of mda-7/IL-24 and IL-24 delE5 (Supplementary Fig. S3A and B).

These results strongly suggest that TPA induces mda-7/IL-24 and IL-24 delE5 expression by activating the MAPK/ERK pathway during U937 and HL60 cell monocytic differentiation.

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Ectopic overexpression of mda-7/IL-24 and IL-24 delE5 induces differentiation of acute myeloid leukemic cells

Our previous study showed that none of the hematopoietic malignant cell lines tested, including U937 and HL60, expressed mda-7/IL-24 intact receptor pairs, and recombinant mda-7/IL-24 had no effect on these cell lines (14). To evaluate functional consequences of mda-7/IL-24 and IL-24 delE5 induction by TPA, mda-7/IL-24 and IL-24 delE5 were transiently transfected into U937 and HL60 cells respectively. Prior to the start of our study, a serial amount of these plasmids were transiently transfected into U937 and HL60 cells, and a dose-dependent increase in transfection and gene expression using less than 1 μg of plasmid was observed. Thus, 1 μg plasmid was used in all subsequent transfection experiments.

A transfection efficiency of 70% and 65% for U937 and HL60 cells, respectively, was typically observed using fluorescence microscopy. For each cell line, cells transfected with mda-7/IL-24 or IL-24 delE5 contained comparable expression of mda-7/IL-24 and IL-24 delE5 protein (Fig. 4A). Transient transfection with mda-7/IL-24 or IL-24 delE5 in U937 and HL60 cells resulted in remarkably upregulated expression of the mature myeloid cell surface proteins CD11b and CD14 ($p < 0.05$), as compared with controls (Fig. 4B). The expression of monocyte/macrophage surface marker CD115 was also increased in U937 cells, but not in HL60 cells (Fig. 4B). In addition, a significant inhibition in cell growth, both in vitro and in vivo ($p < 0.001$; Supplementary Fig. S4A and B), and a significant decrease in the number and size of colonies ($p < 0.001$; Supplementary Fig. S4C) were observed,

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but no significant induction of apoptosis was detected ($p > 0.05$; Supplementary Fig. S5).

These data suggest that ectopic overexpression of mda-7/IL-24 and IL-24 delE5

significantly induces differentiation and inhibits growth of U937 and HL60 cells.

To further investigate whether induction of differentiation also occurs in primary leukemic

cells, we next tested 3 patient samples with acute monocytic leukemia. BMMCs were

obtained, and transiently transfected with mda-7/IL-24 or IL-24 delE5. In all of the leukemia

cells tested in the present study, ectopic overexpression of mda-7/IL-24 or IL-24 delE5

induce morphological evidence of differentiation, including nuclear condensation and

shifting of the nuclear/cytoplasmic ratio from >1 to <1 (Fig. 4C). The expression of CD11b,

CD14 and CD115 was also enhanced to varying degrees ($p < 0.05$; Fig. 4D). These results

suggested mda-7/IL-24 and IL-24 delE5 were able to induce differentiation of blasts from

patients with AML. However, as in the U937 and HL60 cell lines, no significant induction of

apoptosis was observed (data not shown).

mda-7/IL-24 and IL-24 delE5 have no effect on normal hematopoietic progenitors

TPA has been shown to inhibit the colony formation of both leukemic progenitor cells

(L-CFU) and normal myeloid colony (CFU-C) (17). In our study, mda-7/IL-24 and IL-24

delE5 were shown to induce differentiation of leukemic cells. Thus, we next asked whether

these genes affected normal hematopoietic progenitor cells. To address this issue, CD34^{+}

cells were purified from normal cord blood and transfected with mda-7/IL-24 or IL-24 delE5.

A transfection efficiency of 60% was typically observed. Measurement of colony formation

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and the liquid culture assay showed that no significant difference in colony number, morphology, or the expression level of monocytic surface markers was observed as compared with control (p > 0.05; Fig. 5A and B), suggesting that mda-7/IL-24 and IL-24 delE5 have no cytopathic and differentiation-inducing effect on normal hematopoietic precursors. Notably, no colonies or clusters were found when CD34$^+$ cells were treated with TPA at concentrations of 100 nM, 50 nM, 25 nM, 10 nM, 5 nM, 2 nM, or 1 nM (data not shown).

mda-7/IL-24 and IL-24 delE5 induce differentiation of leukemic cells by promoting ROS production

Reactive oxygen species (ROS) regulate apoptosis and proliferation in response to a variety of stimuli (18), and it also play important roles in cell differentiation and senescence (19). An intriguing question is whether ROS can also mediate the differentiation of leukemic cells induced by mda-7/IL-24 and IL-24 delE5. To test this hypothesis, U937, HL60 cells and blasts from patients with AML-M5 transiently transfected with mda-7/IL-24 or IL-24 delE5 were stained for ROS production using H2DCF-DA. We observed mda-7/IL-24 or IL-24 delE5 increased the level of ROS in a time-dependent manner at 12 hours, 24 hours and 48 hours after transfection (data not shown), and the most prominent increase of ROS was at 48 hours ($p < 0.05$; Fig. 6A).

To further study whether ROS is necessary for mda-7/IL-24- and IL-24 delE5-mediated differentiation, U937 and HL60 cells were treated with 25 μM of NAC after transfection.

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Treatment with NAC significantly inhibited the upregulation of CD11b, CD14 and CD115 induced by mda-7/IL-24 or IL-24 delE5 ($p < 0.05$; Fig. 6B), indicating a requirement for ROS in mda-7/IL-24- and IL-24 delE5-mediated differentiation in U937 and HL60 cells.

The predominant intracellular source of ROS generation in monocytes is NADPH oxidase (20, 21). To analyze the source of ROS in the leukemic cells transfected with mda-7/IL-24 or IL-24 delE5, the effect of DPI, a well-known inhibitor of NADPH oxidase, on ROS production was examined. As shown in the Fig. 6C, the increase of ROS induced by mda-7/IL-24 and IL-24 delE5 was abrogated by 10 µM DPI. In addition, enzymatic activity assay showed that mda-7/IL-24 or IL-24 delE5 significantly increased NADPH oxidase activity in U937 and HL60 cells (Fig. 6D). These results indicate that ROS induced by mda-7/IL-24 and IL-24 delE5 is mainly generated by NADPH oxidase.

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Discussion

Defining changes in gene expression occurring during induction of growth arrest and terminal differentiation in human cancers permits identification of genes that may regulate tumor growth and differentiation (22). Here, we observed that the expression of not only mda-7/IL-24 but also IL-24 delE5, which is a splice variant of mda-7/IL-24, was induced when the two myeloid leukemic cell lines U937 and HL60 were induced to differentiate into monocytes following treatment with TPA. The expression of mda-7/IL-24 in monocytic-differentiated U937 and HL60 cells is reminiscent of what was observed in highly differentiated/activated CLL B cells and terminally differentiated melanocytes (2, 23). Knockdown of mda-7/IL-24 and IL-24 delE5 with short interfering RNA resulted in significant inhibition of TPA-induced monocytic differentiation, indicating induction of mda-7/IL-24 and IL-24 delE5 contributes to the monocytic differentiation in leukemia cells mediated by TPA. Additionally, we showed that TPA increased phosphorylation of ERK, and the ERK inhibitors U0126 and PD98059 not only inhibited the induction of mda-7/IL-24 and IL-24 delE5 expression, but also blocked monocytic differentiation in TPA-treated U937 and HL60 cells. Another laboratory has described a transient activation of the ERK1/2 pathway in differentiating HL60 cells treated with 1,25 dihudroxyvitamin D (24). These results suggest that activating the MAPK/ERK pathway might be a common event during leukemic cells monocytic differentiation induced by different differentiation-inducing agents. Our findings reveal a novel mechanism by which TPA induces monocytic differentiation. The individual effect of mda-7/IL-24 and IL-24

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delE5 remains to be determined. Such analysis is currently hampered by the lack of an experimental strategy to individually silence each of the two mRNAs, because the two transcripts exhibit high homology.

To evaluate functional consequences of mda-7/IL-24 and IL-24 delE5, we examined the effect of mda-7/IL-24 and IL-24 delE5 on leukemic cells differentiation and proliferation. Our results suggested that ectoptic overexpression of mda-7/IL-24 or IL-24 delE5 significantly induced differentiation of the myeloid leukemia cell lines and blast cells without causing significant apoptosis, but we cannot rule out that mda-7/IL-24 or IL-24 delE5 have other effects such as autophagy. The expression of monocyte/macrophage surface marker CD115 was increased in U937 cells, but not in HL60 cells. It may be presumed that the cell lineage commitment and differentiation is dependent on the developmental level of the cell, rather than the types of differentiation regulator. mda-7/IL-24 has been described to induce apoptosis of B-lymphoblastic leukemia (25). Our results show that mda-7/IL-24 inhibits AML growth and induce AML differentiation. However, Sainz Perez et al report that high mda-7/IL-24 expression promotes malignant cell survival in CLL (23). One possible explanation for the diversity is that the effect of mda-7/IL-24 on leukemia is dependent on leukemia subtype.

Ectoptic overexpression of mda-7/IL-24 or IL-24 delE5 significantly inhibited the growth of U937 and HL60 cells both in vitro and in vivo. We found no statistically significant difference in all the antileukemia characteristics between mda-7/IL-24 and IL-24 delE5 (p>0.05), suggesting that IL-24 delE5 functions similarly as mda-7/IL-24. Thus, for the first

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time, we show that IL-24 delE5 exerts antileukemia effect in AML cells. Because the

expression of IL-24 delE5 was always accompanied by full-length mda-7/IL-24 expression

in U937 and HL60 cells treated with TPA, to rule out the possibility that functional activity

requires coexistence of the two proteins, we also constructed a plasmid carrying both

mda-7/IL-24 and IL-24 delE5 genes. We observed that the antitumor activity induced by the

construct coexpressing mda-7/IL-24 and IL-24 delE5 was higher than that induced by

mda-7/IL-24 or IL-24 delE5, but no synergistic effect could be found (data not shown).

TPA is an extraordinarily potent stimulator of differentiation of cultured human

promyelocytic leukemia cells (26, 27). However, clinical use of TPA has been hampered by

the fact that, as a multifunctional substance, TPA is also a tumor promoter and

proinflammatory agent, and it can cause serious adverse events in patients (28). In our

study, no colonies or clusters were observed following treatment with as little as 1 nM TPA

in CD34$^+$ cells from normal cord blood. In contrast, transfection with the mda-7/IL-24 or

IL-24 delE5 plasmids did not affect morphology, colony formation, or differentiation in

CD34$^+$ normal cells. These results suggest that mda-7/IL-24 and IL-24 delE5 did not elicit

deleterious effects on normal hematopoietic progenitor cells and that the

differentiation-inducing and growth-suppressing activities are leukemia specific.

Since the discovery of ROS, much attention has focused on the oxidative damage

elicited by ROS within cells. However, recent studies have shown that ROS can act as

signaling molecules that are involved in cell differentiation (29-31). Exogenously added

antioxidants have been shown to block the monocytic differentiation induced by the

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combination of superoxide ions and platelet-activating factor in rat monocytic leukemia c-WRT-LR cells (32). These findings led us to speculate that ectopic overexpression of mda-7/IL-24 and IL-24 delE5 induces ROS production in leukemic cells. In agreement with this hypothesis, our results showed that transfection of mda-7/IL-24 or IL-24 delE5 in U937 and HL60 cells and blast cells increased ROS levels, and ROS was found to be required for cell differentiation. Furthermore, we observed that the increased ROS was mainly generated by NADPH oxidase. It has been reported that blasts isolated from patients with various types of leukemia showed a significant increase of the oxygen radicals in the malignant cells compared to normal leukocytes, and leukemic cells is intrinsically under oxidative stress and therefore more vulnerable to further stress (33, 34). mda-7/IL-24 and IL-24 delE5 leading to severe further ROS accumulation may increase the ROS levels beyond the threshold of tolerability in leukemia cells, resulting in differentiation of leukemic cells, while sparing normal cells.

In conclusion, our study determines the mechanism underlying the monocytic differentiation effect of TPA in human myeloid leukemic cells. We show that the induction of mda-7/IL-24 and IL-24 delE5 contributes to TPA-induced monocytic differentiation, and the MAPK/ERK pathway is required for the induction of mda-7/IL-24 and IL-24 delE5. In addition, we show for the first time the specific differentiation induction roles of mda-7/IL-24 and IL-24 delE5 in leukemic cells, and they can perform a similar differentiation-inducing function to TPA without similar cytopathic problems. These observations bring new insights into the regulation of AML cell differentiation.

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Figure legends

Figure 1. TPA induces the expression of mda-7/IL-24 and IL-24 delE5 in U937 and HL60 cell lines. U937 and HL60 cells were treated with 20 nM TPA for 0, 24, 48, or 72 hours. (A) RT-PCR was performed using primers for mda-7/IL-24 and GAPDH. (B) The relative expression levels of mda-7/IL-24 and IL-24 delE5 were analyzed by quantitative real-time RT-PCR in U937 and HL60 cells treated with TPA for 48 hour. Threshold cycle (Ct) of amplified products, which were amplified using primers specific to mda-7/IL-24 and IL-24 delE5, was shown. (C) The expression of mda-7/IL-24 and IL-24 delE5 protein was assessed by western blotting. Representative results from three independent experiments are shown.

Figure 2. RNA interference of the mda-7/IL-24 and IL-24 delE5 gene blocks TPA-induced monocytic differentiation of U937 and HL60 cells. U937 and HL60 cells were transfected with 100 nM siRNA corresponding to mda-7/IL-24, IL-24 delE5, or non-targeting siRNA (NT). After transfection, differentiation was induced by addition of TPA. (A) After 48 hours, the expression of mda-7/IL-24 and IL-24 delE5 protein were assessed by western blotting, Bands were quantified by scanning densitometry using an LKB Ultrascan XL Laser Densitometer (Kodak Ltd, Hemel Hempstead, UK). (B) After 72 hours, monocytic surface markers CD11b, CD14 and CD115 were analyzed by FACS. Results were expressed as the mean ± S.D. of three independent experiments ($p < 0.05$).

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Figure 3. Regulation of TPA-induced mda-7/IL-24 and IL-24 delE5 expression and differentiation through the MAPK/ERK pathway in U937 and HL60 cells. U937 and HL60 cells were pretreated with or without the MEK inhibitor U0126 or PD98059 for 30 minutes and subsequently exposed to TPA for 72 hours. (A-B) The phosphorylated, total ERK1/2, and mda-7/IL-24 and IL-24 delE5 proteins were determined by western blotting using specific antibodies. (C) The expression of CD11b, CD14, and CD115 was estimated by FACS analysis. Data were presented as mean ± S.D. of three independent experiments (p < 0.001). (D) Cellular morphological changes associated with differentiation were detected with Wright-Giemsa staining. Representative micrographs from three independent experiments with similar results are shown (Leica DM4000B microscope, 40×/0.75 HCL PL objective lens, Leica DC500 digital camera. Original magnifications ×200 (i, iii) ×1 000(ii, iv)).

Figure 4. Ectopic overexpression of mda-7/IL-24 and IL-24 delE5 induces differentiation of leukemic cells. U937 and HL60 cells and blasts from 3 patients with AML-M5 were transfected with mda-7/IL-24, IL-24 delE5, or the empty vector as a negative control. (A) After 48 hours, the expression of mda-7/IL-24 and IL-24 delE5 protein was detected by western blotting in U937 and HL60 cells. (B) After 72 hours, the expression of CD11b, CD14 and CD115 in U937 and HL60 cells was assessed using FACS. Results were expressed as the mean ± S.D. of three independent experiments (p < 0.05). (C) After 72 hours, the morphological changes in blasts from AML patients were analyzed with

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Wright-Giemsa staining. Arrows indicate the differentiated cells with nuclear condensation and decreased nuclear to cytoplasmic ratio. (Leica DM4000B microscope, 40×/0.75 HCL PL objective lens, Leica DC500 digital camera. Original magnifications × 1 000). (D) The expression of CD11b, CD14 and CD115 in blast cells was assessed using FACS.

Figure 5. mda-7/IL-24 and IL-24 delE5 have no effect on normal hematopoietic progenitors. Purified CD34$^+$ cells were transfected with mda-7/IL-24 or IL-24 delE5. (A) The colonies of CFU-GM, BFU-E, and CFU-GEMM were scored. Results were expressed as the mean ± S.E. of three independent experiments ($p > 0.05$). (B) The expression of CD11b, CD14, and CD115 on cells cultured in liquid medium was detected using FACS on the indicated days. Data were presented as mean ± S.D. of three independent experiments ($p < 0.05$).

Figure 6. mda-7/IL-24 and IL-24 delE5 induce differentiation of leukemic cells by inducing ROS production. U937, HL60 cells and blasts from patients with AML-M5 were transfected with mda-7/IL-24 or IL-24 delE5. (A) After transfection, cells were cultured for 48 hours, ROS was monitored by FACS. Data were presented as mean ± S.D. of three independent experiments ($p < 0.05$). (B) After transfection, cells were cultured in medium with or without 25 µM NAC for 72 hours. The expression of CD11b, CD14 and CD115 was assessed using FACS. Results were expressed as the mean ± S.D. of three independent experiments from each cell line ($p < 0.05$). (C) After transfection, cells were cultured in medium with or without 10 µM DPI for 48 hours, ROS was monitored by FACS.

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Data were presented as mean ± S.D. of three independent experiments ($p < 0.05$). (D)

NADPH oxidase activity was measured 48 hours after transfection. Data represent the

mean ± S.E.; $p < 0.05$, compared with vector control. Data are representative of at least
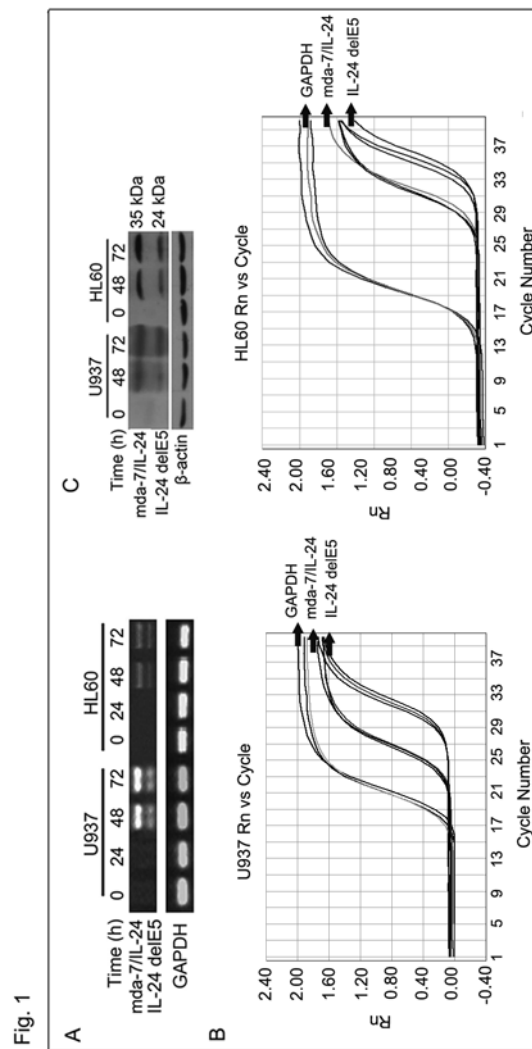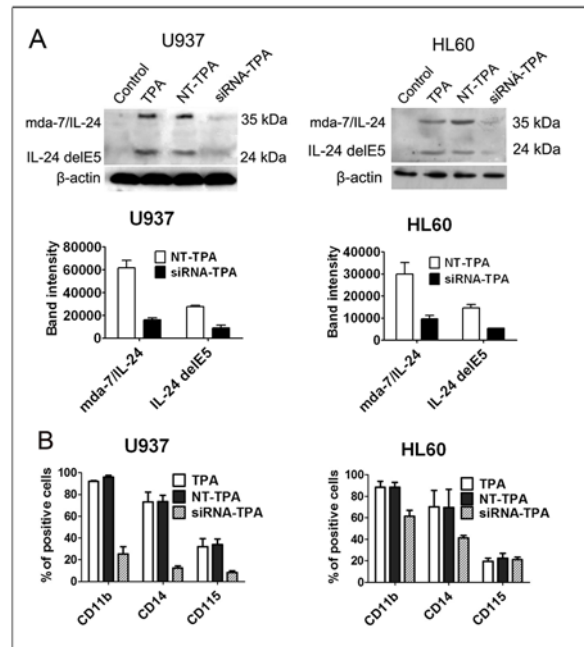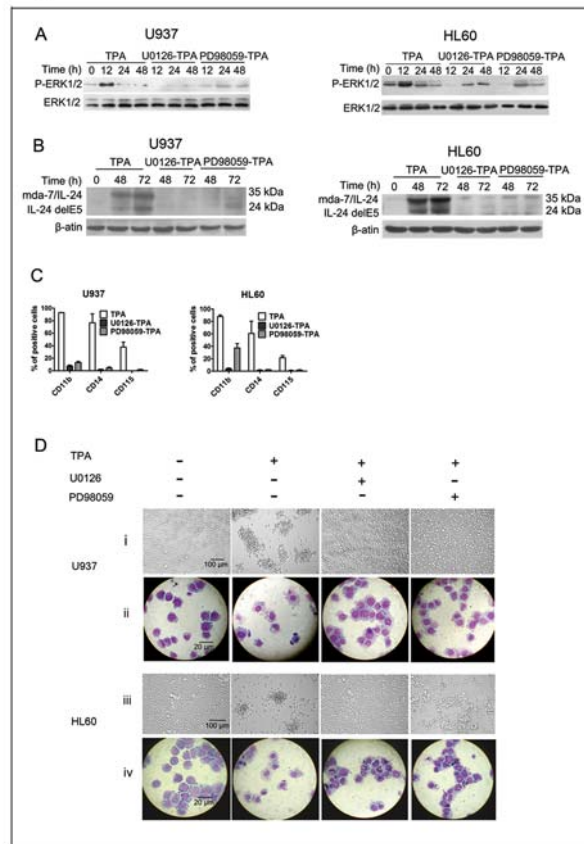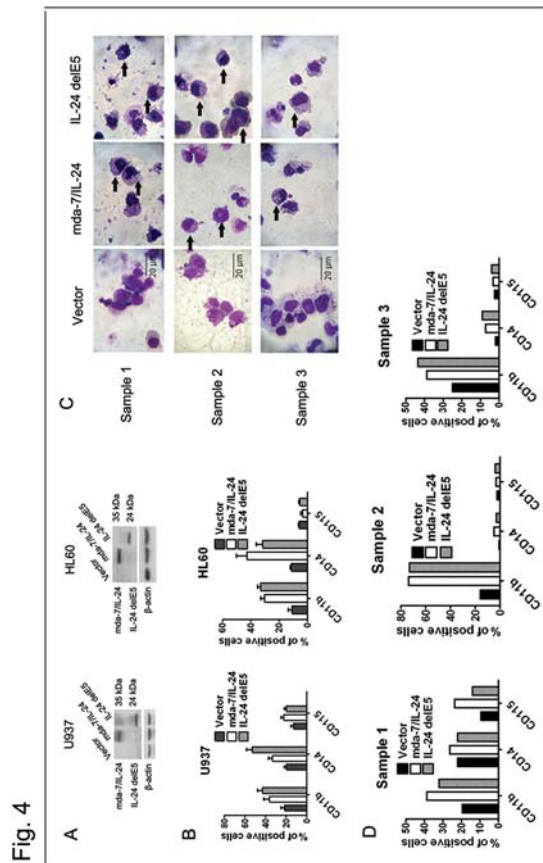
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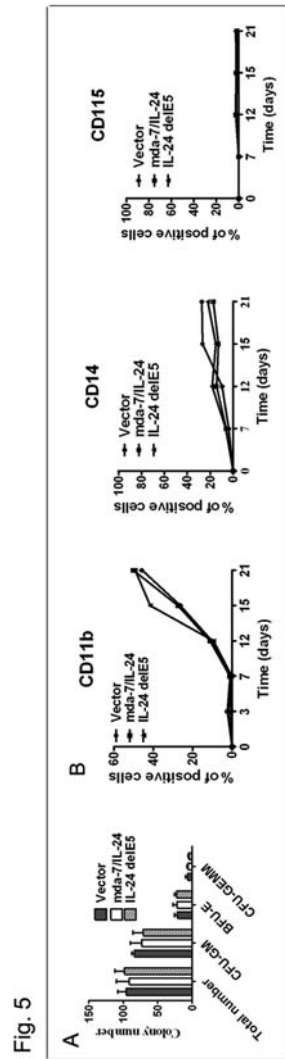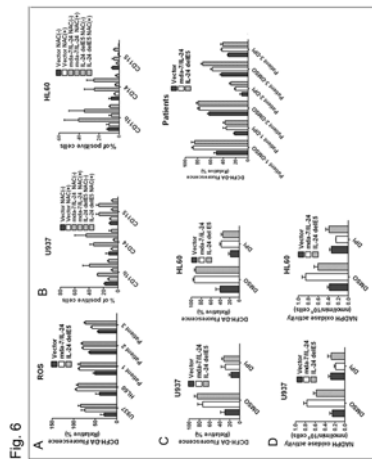
Fig. 1

Fig. 2

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Fig. 5

Fig. 6

Molecular Cancer Therapeutics

Novel functions for mda-7/IL-24 and IL-24 delE5: regulation of differentiation of acute myeloid leukemic cells

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