Published in final edited form as:
Exp Cell Res. 2010 April 15; 316(7): 1254–1262. doi:10.1016/j.yexcr.2010.01.007.

Up- or down-regulation of tescalcin in HL-60 cells is associated with their differentiation to either granulocytic or macrophage-like lineage

Konstantin Levay[1,*] and **Vladlen Z. Slepak**[1,2]

[1]Department of Molecular and Cellular Pharmacology, University of Miami Miller School of Medicine, Miami, FL, 33136

[2]Neuroscience Program, University of Miami Miller School of Medicine, Miami, FL, 33136

Abstract

Tescalcin is a 25 KDa EF-hand Ca^{2+}-binding protein that is differentially expressed in several mammalian tissues. Previous studies demonstrated that expression of this protein is essential for differentiation of hematopoietic precursor cell lines and primary stem cells into megakaryocytes. Here we show that tescalcin is expressed in primary human granulocytes and is upregulated in human promyelocytic leukemia HL-60 cells that have been induced to differentiate along the granulocytic lineage. However, during induced macrophage-like differentiation of HL-60 cells the expression of tescalcin is down-regulated. The decrease in expression is associated with a rapid drop in tescalcin mRNA level, whereas upregulation occurs via a post-transcriptional mechanism. Tescalcin is necessary for HL-60 differentiation into granulocytes as its knockdown by shRNA impairs the ability of HL-60 cells to acquire the characteristic phenotypes such as phagocytic activity and generation of reactive oxygen species measured by respiratory burst assay. Both up- and down-regulation of tescalcin require activation of the MEK/ERK cascade. It appears that commitment of HL-60 cells toward granulocytic versus macrophage-like lineage correlates with expression of tescalcin and kinetics of ERK activation. In retinoic acid-induced granulocytic differentiation the activation of ERK and upregulation of tescalcin occurs slowly (16-48 hours). In contrast, in PMA-induced macrophage-like differentiation the activation of ERK is rapid (15-30 minutes) and tescalcin is down-regulated. These studies indicate that tescalcin is one of the key gene products that is involved in switching differentiation program in some cell types.

Introduction

HL-60 cell line is an established *in vitro* model to study cellular differentiation and signal transduction. These cells were originally isolated from a patient with acute myeloblastic leukemia with maturation, FAB-M2 [1,2]. HL-60 cells can be induced to terminally differentiated granulocytes or monocytes/macrophages in response to a variety of inducers [3-8].

* Address correspondence to: Konstantin Levay, Department of Molecular and Cellular Pharmacology, University of Miami Miller School of Medicine, P. O. Box 016189 (R-189), Miami, FL 33101-6189 Ph: (305) 243-3431; Fax: (305) 243-4555; klevay@med.miami.edu.

Previous studies demonstrated that differentiation of HL-60 cells requires sustained activation of ERK-1 and/or ERK-2 - extracellular signal-regulated kinases that belong to the mitogen-activated protein kinase (MAPK) family. ERK-1 and ERK-2 are activated by highly homologous dual specificity kinases MEK-1 and MEK-2 [9]. Sustained activation of MEKs and ERKs was observed during both cytokine- and chemically-induced myeloid differentiation [10-16]. While it is established that MEK/ERK signaling is essential for myeloid differentiation of hematopoietic cell lines and primary progenitor cells, the exact mechanisms whereby this pathway affects myelopoiesis are incompletely understood. A number of studies suggest that the MEK/ERK/MAPK pathway is central for linking various extracellular ligands to their multiple cellular target proteins that activate myeloid transcription factors and other specific mechanisms that promote differentiation [13,15,16]. For example, activated ERK1 and ERK2 phosphorylate a number of different substrates, including kinase $p90^{RSK}$, Ets family transcriptional factor Elk-1, AP-1, c-Myc, and STATs [17-21].

Tescalcin was discovered as an autosomal gene that is differentially expressed in embryonic gonads [22]. This conserved gene encodes a 24-kDa protein with a single functional EF-hand domain that can bind Ca^{2+} with micromolar affinity [23,24]. In vitro studies showed that tescalcin can interact with cytoplasmic tail of Na^+/H^+ exchanger [25-28] and can inhibit the phosphatase activity of Calcineurin A [23]. However, whether these observations might relate to the in vivo functions of tescalcin is not known. Tescalcin is expressed predominantly in the mouse heart, brain, stomach and testis, as well as in mouse and human primary hematopoietic progenitor cells and cell lines [12,23]. During differentiation and maturation of megakaryocytes the expression of tescalcin is dramatically increased upon sustained activation of ERK-1/-2. Furthermore, tescalcin was shown to be a critical factor in megakaryocytic differentiation that is necessary for coupling MEK/ERK cascade with expression of Ets family transcription factors [12].

In this paper we examined the expression of tescalcin in HL-60 cells and found that it was transcriptionally and post-transcriptionally regulated during induced differentiation of these cells and that it was required for optimal granulocytic maturation.

Material and Methods

Materials

Rabbit polyclonal antibody against GAPDH (sc-25778) and mouse monoclonal antibody against β-actin (MAB1501R) were obtained from Santa Cruz Biotechnology and Millipore, respectively. All-*trans* retinoic acid, PMA, nitro blue tetrazolium (NBT), Wright-Giemsa stain and α-naphtyl acetate esterase staining kit were purchased from Sigma-Aldrich. MEK-specific inhibitors U0126 and PD98059, antibodies to p44/42 MAPK, and Phospho-p44/42 MAPK (Thr202/Tyr204) were from Cell Signaling Technologies. The yellow-green fluorescent (505/515) 1.0 μm carboxylate-modified FluoSpheres® beads were purchased from Invitrogen. Protease inhibitor cocktail (Complete, EDTA-free; Roche) was supplemented in all cellular lysates. Protein electrophoresis reagents and markers were from Bio-Rad. Cell culture plastic and media were purchased from BD Falcon. Other chemicals were from Sigma-Aldrich. Secondary anti-mouse and anti-rabbit antibodies were obtained from Jackson Immunologicals. Lympholyte-poly™ granulocyte isolation media was obtained from Cedarlane Laboratories. Red Blood Cell lysis buffer was purchased from Roche Diagnostics. Human Serum Albumin was obtained from Sera Care Life Sciences.

Production of a mouse monoclonal antibody against tescalcin

Recombinant full-length tescalcin was expressed and purified from E. coli according to established protocol [23]. Two BALB/c mice were injected intraperitoneally with 100 μg of

tescalcin in a total volume of 200 μl of 1:1 emulsion with saline adjuvant. Injection was repeated twice with 14 days interval. Three days before the day of fusion, mice were boosted via the tail vein; spleens were removed and desegregated into a single cell suspension. Resulting splenocytes were fused to a parental SP2/0 myeloma cells and plated in ClonaCell-HY hybridoma selective medium (StemCell Technologies). Ten to fourteen days later, approximately 1000 colonies were picked and placed into individual wells of a 96-well tissue culture plates containing ClonaCell-HY growth medium. In the first round of screening, the reactivity against tescalcin in hybridoma supernatants was analyzed by ELISA. Positive hybridoma clones were further expanded and their ability to detect tescalcin was analyzed by Western blot. Hybridoma clone 4D94 was selected for the antibody production.

Granulocyte Isolation

Human polymorphonuclear granulocytes were isolated from venous blood, taken by venepuncture from healthy consenting volunteers, on Lympholyte-poly™ separation media as described [29]. Briefly, blood was drawn and dispensed immediately into 15 ml sterile polypropylene centrifuge tubes containing heparine and mixed gently. Five ml of blood were then carefully layered over equal volume of separation media and centrifuged at 500 g and 22° C for 35 min. The layer containing polymorphonuclear (PMN) cells was removed into the fresh tube and diluted to 10 ml with HBSS without Ca^{2+}/Mg^{2+} (Invitrogen). Cell suspension was centrifuged at 350 g and 22°C for 10 min and the supernatant was discarded. Residual red blood cells (RBCs) were lysed by gentle resuspension of a pellet in 2 ml of the RBC Lysis buffer (Roche) and cells were centrifuged at 250 g for 5 min. Pelleted cells were washed once in HBSS without Ca^{2+}/Mg^{2+} and resuspended in HBSS with 2% human serum albumin for further analysis. Granulocyte preparations were assessed for purity using a commercial Wright-Giemsa staining kit (Sigma-Aldrich) and were found to contain >95% granulocytes.

Sample Preparation and Western Blot Analysis

Exponentially growing suspension HL-60 cells were collected by centrifugation (300g, 4°C, 10 min.) and the cell pellet was washed twice with ice cold PBS. To obtain total cell lysate the pellet was gently resuspended in a lysis buffer (20 mM Tris-HCl pH7.5; 150 mM NaCl; 1 mM Na_2EDTA; 1 mM EGTA; 1% Triton X-100; 2.5 mM $Na_2P_2O_7$; 1 mM beta-glycerophosphate; 1 mM Na_3VO_4; protease inhibitors) and incubated on ice for 30 minutes. Petri dishes with adherent macrophage-like cells were rinsed twice with PBS on ice to remove residual media and cells were gently scraped into lysis buffer. Further homogenization was performed by ultrasonic cell disruptor (Misonix) and the lysate was centrifuged at 15,000g for 15 minutes, 4°C. Protein concentration was measured with Bio-Rad Bradford Protein Assay unless otherwise stated. To prepare sample for gel loading, the total cell lysate was mixed with 5X SDS sample buffer (10% SDS; 62.5 mM Tris-HCl pH6.8; 25% glycerol; 125 mM DTT). Proteins were resolved on 12% PAGE and transferred onto nitrocellulose (Schleicher & Schull). Membranes were blocked in 1% w/v BSA in TBST (20 mM Tris, 50 mM NaCl, and 0.1% Tween-20, pH 7.4), probed with primary antibodies for 1-2 h at room temperature or overnight at 4°C, and then incubated with appropriate horseradish peroxidase-conjugated secondary antibodies. Visualization of protein bands was performed by enhanced chemiluminescence (SuperSignal Western Blotting Kit; Thermo Scientific) and exposure to Kodak X-Omat film.

Cell Culture, Transfection, and Stable Cell Lines

HL-60 cells (CCL-240) and U-937 cells (CRL-1593.2) were supplied by American Type Culture Collection (ATCC). HL-60 cultures were maintained at concentrations between 1×10^5 and 1×10^6 of viable cells/ml at 37°C under 5% CO_2 in air and 95% humidity, in Iscove's modified Dulbecco's medium (IMDM) supplemented with 20% heat-inactivated fetal bovine

serum (FBS) and 1% Penicillin/Streptomycin as recommended by ATCC. U-937 cultures were maintained in RPMI-1640 medium with 10% heat-inactivated FBS and 1% Penicillin/Streptomycin. For transfection, the exponentially growing HL-60 cells were washed twice with PBS and resuspended in serum-free IMDM media at a concentration of 2×10^7 cells/ml. Electroporation was performed using 80 μg of plasmid DNA mixed with 0.8 ml of cell suspension. Cells were subjected to a single electric pulse (960 μF, 250 V) in 0.4-cm gap electroporation cuvettes using a Bio-Rad Gene Pulser electroporator, followed by dilution to 5×10^5 cells/ml in IMDM cell culture media. For making stable cell lines, transfected cells were selected for neomycin resistance in the presence of 800 μg/ml Geneticine (Invitrogen) and then analyzed for the expression of tescalcin by Western blotting. Stable cell lines were routinely maintained in IMDM supplemented with 20% FBS and 500 μg/ml Geneticine.

Induction and assessment of differentiation

Exponentially growing HL-60 cells were seeded in fresh culture medium at 2×10^5 cells/ml supplemented with inducer and cultured for 5-7 days at 37°C in a humidified atmosphere and 5% CO_2. For morphological assessment of the cells, Cytospin slides were stained with Wright-Giemsa and examined under a light microscope at 1000× magnification. Granulocytic differentiation was monitored by NBT reduction that was assayed essentially as described earlier [3]. In brief, cells were washed twice with PBS and then incubated for 25 min at 37°C with 0.1% NBT in PBS containing 100 ng/ml PMA. The percentage of cells with intracellular blue-black formazan deposits was then determined on Wright-Giemsa stained Cytospin slide preparations. At least 400 cells were assessed for each experimental point. Monocyte/macrophage differentiation was assayed using non-specific α-naphthyl acetate esterase staining kit (Sigma-Aldrich) according to manufacturer's recommended protocol.

Phagocytosis assay

HL-60 cells were cultured with 10^{-6} M retinoic acid or 0.1% DMSO for 72 hours. Treated cells were washed and incubated in fresh culture media containing 0.025% of the yellow-green fluorescent (505/515) 1 μm carboxylate-modified microspheres for 30 min at 37°C. Control cultures with no addition of microspheres were also included. The cell were then washed five times in cold PBS to eliminate uningested particles and suspended in PBS for the analysis. Fluorescence derived from microspheres ingested by the cells was immediately measured using FACScan fluorescence-activated cell sorter and CELLQuest analytical software (Becton Dickinson). Laser excitation at 488nm and emission of 530/30 nm band pass was used for detection. Only live cells were gated using forward and side scatter patterns. Untreated cells were used to establish the autofluorescence intensity background. Assays were performed in triplicates.

RNA isolation and quantification of gene expression

Total RNA was extracted from cells using Trizol reagent (Invitrogen), purified over RNeasy column with DNase digestion step (QIAGEN), and converted to cDNA using High-Capacity Reverse Transcription kit (Applied Biosystems). Real-time quantitative PCR (qPCR) was performed using 100 ng of cDNA, Taqman Fast Universal PCR Master Mix, and the following Taqman Gene Expression Assays: Tescalcin (Hs00215487_m1), c-Myc (Hs00905030_m1), CD38 (Hs00277045_m1), and 18S (4333760T). The reactions were run in triplicates on the 7900HT Fast Real-Time PCR System (Applied Biosystems) and normalized to the endogenous control 18S rRNA.

Statistical analysis

Statistical analysis was performed using paired, two-tailed Student's *t* test and GraphPad QuickCalcs software. Statistical significance was accepted at the $p < 0.05$ level.

Results and Discussion

We showed earlier that tescalcin is essential for hematopoietic stem cell differentiation toward megakaryocytic lineage [12]. Tescalcin is also expressed in human leukemia HL-60 cells [23], an established model cell line that can be differentiated *in vitro* toward either granulocytes or monocytes-macrophages [5,8]. We investigated if tescalcin is involved in these processes.

Tescalcin level changes during differentiation of HL-60 cells

To induce granulocytic differentiation, we cultured cells for 5 days in the presence of all-trans retinoic acid (ATRA), dimethylformamide (DMF) or dimethyl sulfoxide (DMSO) [3,5]. Granulocytic differentiation was tested by reduction of nitro blue tetrazolim (NBT). In addition, the change in expression of characteristic marker mRNAs was confirmed by qPCR. For the induction of macrophage-like differentiation, HL-60 cells were cultured for 5 days in the presence of 16 nM PMA [8] and then tested for non-specific esterase activity. Treatment with PMA also resulted in strong adherence to plastic, which is characteristic for HL-60 cells undergoing macrophage differentiation [8,30]. Western blot analysis of total cell lysates revealed that treatment with ATRA, DMF, or DMSO caused the level of tescalcin to increase 5-10-fold (Fig. 1A). To test if tescalcin is expressed in mature granulocytes, we isolated human polymorphonuclear granulocytes from peripheral blood. Western blot analysis revealed a single 25 KDa band corresponding to tescalcin (Fig. 1B). The presence of tescalcin in primary granulocytes is consistent with tescalcin upregulation during granulocytic differentiation. In contrast, during the PMA-induced monocytic-macrophage differentiation of HL-60 cells the expression of tescalcin dropped below the detection level (Fig. 2). In another cell line, U-937, which is also known as an *in vitro* model for the monocyte/macrophage differentiation [31] treatment with PMA resulted in a similar downregulation of tescalcin (Fig. 2). On the basis of these results, we concluded that differentiation of HL-60 cells into different lineages is associated with the opposing effects on tescalcin expression. Next, we investigated kinetics and mechanisms of these phenomena.

Upregulation of tescalcin in HL-60 cells

Following a single application of ATRA, the increase in tescalcin protein level became apparent within 24 hours and reached its maximum at approximately 72 hours (Fig. 3). The timing of tescalcin upregulation coincided with activation of ERK/MAP kinases, as detected by antibodies against phosphorylated forms of ERK1 and ERK2. We found that p42 (ERK2) was the predominantly phosphorylated isoform, consistent with the previous reports of ERK2 being the main species in HL-60 cells [16]. The onset of ERK activation began after 8-16 hours of ATRA treatment and the activation persisted for up to 72 hours. Similar results have been reported in other studies [13,16]. While ERK phosphorylation could no longer be detected after 72 hours, the level of tescalcin remained high for up to 120 hours (Fig.3). Treatment of cells with other agents inducing granulocytic differentiation of HL-60 cells, such as DMSO and DMF, resulted in a similar time-course for tescalcin upregulation as was obtained upon treatment with ATRA (data not shown). These experiments indicated that ERK activation precedes and is possibly required for tescalcin upregulation.

To test if activation of ERK was necessary for the upregulation of tescalcin, we stimulated HL-60 cells with ATRA in the presence of either U0126 or PD98059 -noncompetitive inhibitors of MEKs; these kinases are responsible for the activation of ERKs [32]. Both inhibitors bind specifically to inactive form of MEK1 and/or MEK2 and prevent their activation by phosphorylation [32-34]. In our experiments we used U0126 at 4 μM and PD98059 at 10 μM since these concentrations did not produce an apparent loss in cell viability and growth. Cells were pre-incubated in the presence or absence of these inhibitors for 30 minutes and then stimulated with 1μM ATRA. Three days later cells were collected and analyzed for tescalcin

by immunoblotting. As shown in Fig. 4, both of these inhibitors prevented the increase in tescalcin level. Thus, we can conclude that upregulation of tescalcin in HL-60 cells occurs during ATRA-induced granulocytic differentiation and is mediated by activation of ERK.

Upregulation of tescalcin during granulopoiesis occurs at the post-transcriptional level

Our previous study showed that in the process of megakaryocytic differentiation the level of tescalcin mRNA increased several fold [12]. Here, we used qPCR to test whether induction of granulocytic differentiation in HL-60 cells also leads to the increase in tescalcin mRNA (Fig. 5). Previous studies demonstrated that ATRA-induced granulocytic differentiation of HL-60 cells is associated with the dramatic changes in expression of a number of genes, including CD38 and c-Myc, hence we analyzed these marker mRNAs in our experiments. CD38 gene encodes a glycoprotein that functions as a bifunctional ectoenzyme in the synthesis and hydrolysis of cyclic ADP-ribose and therefore takes part in Ca^{2+} signaling and cell adhesion. The upregulation of CD38 is mediated by retinoic acid receptor (RAR alpha) signaling and plays a causal role in granulocytic differentiation of HL-60 cells [35-38]. The c-Myc proto-oncogene controls key cellular functions including DNA replication, cell cycle progression, differentiation, and growth [39-42]. During granulocytic differentiation of HL-60 cells the level of c-Myc mRNA was shown to be significantly decreased [35,43,44]. Conversely, overexpression of c-Myc is known to antagonize granulocytic differentiation in myeloid progenitors [45]. Contrary to our expectations, the mRNA level of tescalcin did not increase at all during 120 hours post stimulation (Fig. 5). At the same time the expression of CD38 mRNA increased over 50-fold, peaking at 16 hours. Accordingly, steady-state mRNA level of c-Myc dropped 5-fold within the first hour following the ATRA treatment and then decreased 33-fold by the end of experiment. From these results, we conclude that in HL-60 cells undergoing granulocytic differentiation the expression of tescalcin is controlled at the post-transcriptional level.

Our previous study showed that the signal-induced increase in tescalcin expression in primary hematopoietic precursor cells and erythroleukemia K562 cells was necessary for induced megakaryocytic differentiation [12]. Therefore, we hypothesized that upregulation of tescalcin during ATRA-induced granulocytic differentiation of HL-60 cells could play a role in this process.

Knockdown of tescalcin inhibits granulocytic differentiation of HL-60 cells

To test the idea that tescalcin was essential for the ATRA-induced granulocytic differentiation we characterized HL-60 cells where tescalcin was depleted using specific shRNA. Cells were electroporated with shRNA expression plasmids [12] and selected for neomycin resistance. After expanding the transfected cell population, we confirmed the knockdown by Western blotting. The protein level of tescalcin dropped several fold in non-stimulated cells (Fig. 6A) and, accordingly, our qPCR analysis showed a 10-fold decrease in tescalcin mRNA accumulation (Fig. 6B). The treatment with ATRA resulted in only minor increase (Fig. 6A).

Next, we examined the ability of tescalcin-depleted HL-60 cells to differentiate into mature granulocytes (Fig. 7). We used two independent functional assays. In one, the oxidative metabolic burst of activated granulocytes and the superoxide anion-generating capacity of the cells were measured by NBT reduction test. Reduced NBT precipitates where the redox reaction has taken place and is visualized by light microscopy as dark blue granules of formazan inside the cells. Another assay was phagocytosis of fluorescence-labeled microparticles. In both these experiments, tescalcin-depleted HL-60 cells were compared to cells transfected with scrambled shRNA construct, before and after treatment with ATRA. We found that tescalcin knockdown severely impaired the ability of HL-60 cells to differentiate. The number of NBT-positive cells induced by ATRA was reduced 2-fold, and the onset of granulocytic maturation

was delayed by approximately 2 days (Fig. 7A). The number of active phagocytes was reduced 5-fold from 30% in scrambled shRNA-treated HL-60 cells to only 6% in tescalcin-depleted HL-60 cells (Fig. 7B). These results demonstrate that ATRA-induced upregulation of tescalcin is a necessary step in the process of granulocytic differentiation of HL-60 cells.

Downregulation of tescalcin during macrophage differentiation of HL-60 cells

We found that expression of tescalcin in HL-60 cells drops after treatment with PMA (Fig. 2). This suggested that tescalcin level in macrophage differentiation is regulated by a signaling cascade different from the pathway engaged in ATRA-induced granulocytic differentiation, where tescalcin expression is elevated (Fig. 1A). However, it is known that both PMA and ATRA activate MEK/ERK signaling [11,13,16,46]. Furthermore, we found that MEK-1/-2 inhibitor, U0126, blocks PMA-induced downregulation of tescalcin (Fig. 8). This result argues against the possibility that an alternative, ERK-independent, pathway controls tescalcin level in HL-60. How could activation of the same pathway have opposite effects on tescalcin? To address this question we analyzed the kinetics of tescalcin expression and ERK activation in PMA-treated HL-60 cells.

As we show in Fig. 9, the level of tescalcin began to decline approximately 8 hours after addition of PMA, and by 72 hours tescalcin was essentially undetectable. This downregulation coincided with the reduction of its mRNA which declined significantly within 1 hour and almost disappeared within 24 hours (Fig. 10). These results indicate that the drop in tescalcin level during PMA-induced macrophage differentiation occurs via a rapid decline in mRNA which is different from the post-transcriptional mechanism involved in ATRA-induced granulocytic differentiation.

The activation of ERK by PMA occurred very rapidly and reached its maximum within 15-30 minutes. In 4 hours after the application of PMA phosphorylation of ERK somewhat declined but continued to demonstrate a sustained level of ERK activation for the duration of the experiment (120 h). Our experiments show that although ATRA and PMA both activate ERK, this cascade is activated by PMA much faster than by ATRA. One possible explanation is that kinetics of ERK activation determines the mechanism that controls the switch between up- and down-regulation of tescalcin by ATRA versus PMA. Previous studies showed that the major substrates for phorbol esters in HL-60 cells are either PKCβ or PKCδ, both of which activate Raf/MEK/ERK cascade [13,47-51]. Rapid PKC-mediated activation of Raf/MEK/ERK axis of MAPK cascade by PMA causes a fast shut off of tescalcin gene transcription. The delayed induction of ERK by ATRA may suggest that it is mediated indirectly and likely involves a transcriptional step. The effects of ATRA are primarily mediated by retinoic acid nuclear receptors, RARs and RXRs, ligand-activated transcription factors [52-54]. In development and cell differentiation, these receptors bind to specific DNA target sequences and regulate gene transcription. Other potential regulatory effects of retinoids may include inhibition of AP-1activation, protein retinoylation, or histone acetylation [55-57]. One can speculate that these slower events can lead to mobilization of autocrine regulatory loops and prime the cell for a different response resulting in tescalcin accumulation that did not become apparent until 16 to 24 hours after addition of ATRA (Fig. 3). Our results indicate that the requirement for the upregulation of tescalcin which follows the activation of ERKs further delays the onset of granulocytic differentiation. Indeed, 48 hours is typically needed for ATRA to induce the onset of differentiation and G_0-specific growth arrest [16]. Another idea is that PMA and ATRA are known to activate different isoforms of ERK. Indeed, it was reported that, contrary to ERK2 activation by retinoic acid, PMA causes rapid activation of both ERK1 and ERK2 [13].

In summary, the results presented in this paper further develop our previous findings that highlight the role of tescalcin in megakaryopoiesis [12] by demonstrating that it is also essential in granulocytic differentiation. As in megakaryopoiesis, the level of tescalcin in differentiating

HL-60 is regulated by ERK. The new observations made by the current work are (1) the expression of tescalcin can be regulated both at transcriptional and posttranscriptional level and (2) cellular differentiation can be associated with both up- and down-regulation of tescalcin. These findings underscore the role of tescalcin as one of the molecules that can switch the same progenitor cell to differentiate into alternative directions.

Acknowledgments

We are grateful to Dr. Anthony Caswell for critical reading of the manuscript and James Phillips (UM/SCCC Flow Cytometry Resource) for assistance with data acquisition. This work was supported by the UM Sylvester Cancer Center developmental grant (K.L.), UM SAC pilot grant (K.L.), NIH grants GM 060019 (V.Z.S) and NEI 012982 (V.Z.S.)

References

1. Bennett JM, Catovsky D, Daniel MT, Flandrin G, Galton DA, Gralnick HR, Sultan C. Proposals for the classification of the acute leukaemias. French-American-British (FAB) co-operative group. Br J Haematol 1976;33:451–458. [PubMed: 188440]
2. Dalton WT Jr, Ahearn MJ, McCredie KB, Freireich EJ, Stass SA, Trujillo JM. HL-60 cell line was derived from a patient with FAB-M2 and not FAB-M3. Blood 1988;71:242–247. [PubMed: 3422031]
3. Breitman TR, Selonick SE, Collins SJ. Induction of differentiation of the human promyelocytic leukemia cell line (HL-60) by retinoic acid. Proc Natl Acad Sci U S A 1980;77:2936–2940. [PubMed: 6930676]
4. Collins SJ. The HL-60 promyelocytic leukemia cell line: proliferation, differentiation, and cellular oncogene expression. Blood 1987;70:1233–1244. [PubMed: 3311197]
5. Collins SJ, Ruscetti FW, Gallagher RE, Gallo RC. Terminal differentiation of human promyelocytic leukemia cells induced by dimethyl sulfoxide and other polar compounds. Proc Natl Acad Sci U S A 1978;75:2458–2462. [PubMed: 276884]
6. Miyaura C, Abe E, Kuribayashi T, Tanaka H, Konno K, Nishii Y, Suda T. 1 alpha,25-Dihydroxyvitamin D3 induces differentiation of human myeloid leukemia cells. Biochem Biophys Res Commun 1981;102:937–943. [PubMed: 6946774]
7. Olsson IL, Breitman TR, Gallo RC. Priming of human myeloid leukemic cell lines HL-60 and U-937 with retinoic acid for differentiation effects of cyclic adenosine 3′:5′-monophosphate-inducing agents and a T-lymphocyte-derived differentiation factor. Cancer Res 1982;42:3928–3933. [PubMed: 6286101]
8. Rovera G, Santoli D, Damsky C. Human promyelocytic leukemia cells in culture differentiate into macrophage-like cells when treated with a phorbol diester. Proc Natl Acad Sci U S A 1979;76:2779–2783. [PubMed: 288066]
9. Xu S, Robbins D, Frost J, Dang A, Lange-Carter C, Cobb MH. MEKK1 phosphorylates MEK1 and MEK2 but does not cause activation of mitogen-activated protein kinase. Proc Natl Acad Sci U S A 1995;92:6808–6812. [PubMed: 7624324]
10. Herrera R, Hubbell S, Decker S, Petruzzelli L. A role for the MEK/MAPK pathway in PMA-induced cell cycle arrest: modulation of megakaryocytic differentiation of K562 cells. Exp Cell Res 1998;238:407–414. [PubMed: 9473349]
11. Kharbanda S, Saleem A, Emoto Y, Stone R, Rapp U, Kufe D. Activation of Raf-1 and mitogen-activated protein kinases during monocytic differentiation of human myeloid leukemia cells. J Biol Chem 1994;269:872–878. [PubMed: 8288641]
12. Levay K, Slepak VZ. Tescalcin is an essential factor in megakaryocytic differentiation associated with Ets family gene expression. J Clin Invest 2007;117:2672–2683. [PubMed: 17717601]
13. Miranda MB, McGuire TF, Johnson DE. Importance of MEK-1/-2 signaling in monocytic and granulocytic differentiation of myeloid cell lines. Leukemia 2002;16:683–692. [PubMed: 11960350]
14. Miranda MB, Xu H, Torchia JA, Johnson DE. Cytokine-induced myeloid differentiation is dependent on activation of the MEK/ERK pathway. Leuk Res 2005;29:1293–1306. [PubMed: 16164983]

15. Wang X, Studzinski GP. Activation of extracellular signal-regulated kinases (ERKs) defines the first phase of 1,25-dihydroxyvitamin D3-induced differentiation of HL60 cells. J Cell Biochem 2001;80:471–482. [PubMed: 11169731]

16. Yen A, Roberson MS, Varvayanis S, Lee AT. Retinoic acid induced mitogen-activated protein (MAP)/extracellular signal-regulated kinase (ERK) kinase-dependent MAP kinase activation needed to elicit HL-60 cell differentiation and growth arrest. Cancer Res 1998;58:3163–3172. [PubMed: 9679985]

17. Chung J, Uchida E, Grammer TC, Blenis J. STAT3 serine phosphorylation by ERK-dependent and -independent pathways negatively modulates its tyrosine phosphorylation. Mol Cell Biol 1997;17:6508–6516. [PubMed: 9343414]

18. Hill CS, Treisman R. Transcriptional regulation by extracellular signals: mechanisms and specificity. Cell 1995;80:199–211. [PubMed: 7834740]

19. Karin M. The regulation of AP-1 activity by mitogen-activated protein kinases. J Biol Chem 1995;270:16483–16486. [PubMed: 7622446]

20. Lazar DF, Wiese RJ, Brady MJ, Mastick CC, Waters SB, Yamauchi K, Pessin JE, Cuatrecasas P, Saltiel AR. Mitogen-activated protein kinase kinase inhibition does not block the stimulation of glucose utilization by insulin. J Biol Chem 1995;270:20801–20807. [PubMed: 7657664]

21. Seth A, Gonzalez FA, Gupta S, Raden DL, Davis RJ. Signal transduction within the nucleus by mitogen-activated protein kinase. J Biol Chem 1992;267:24796–24804. [PubMed: 1332967]

22. Perera EM, Martin H, Seeherunvong T, Kos L, Hughes IA, Hawkins JR, Berkovitz GD. Tescalcin, a novel gene encoding a putative EF-hand Ca(2+)-binding protein, Col9a3, and renin are expressed in the mouse testis during the early stages of gonadal differentiation. Endocrinology 2001;142:455–463. [PubMed: 11145610]

23. Gutierrez-Ford C, Levay K, Gomes AV, Perera EM, Som T, Kim YM, Benovic JL, Berkovitz GD, Slepak VZ. Characterization of tescalcin, a novel EF-hand protein with a single Ca2+-binding site: metal-binding properties, localization in tissues and cells, and effect on calcineurin. Biochemistry 2003;42:14553–14565. [PubMed: 14661968]

24. Bao Y, Hudson QJ, Perera EM, Akan L, Tobet SA, Smith CA, Sinclair AH, Berkovitz GD. Expression and evolutionary conservation of the tescalcin gene during development. Gene Expr Patterns 2009;9:273–281. [PubMed: 19345287]

25. Li X, Liu Y, Kay CM, Muller-Esterl W, Fliegel L. The Na+/H+ exchanger cytoplasmic tail: structure, function, and interactions with tescalcin. Biochemistry 2003;42:7448–7456. [PubMed: 12809501]

26. Mailander J, Muller-Esterl W, Dedio J. Human homolog of mouse tescalcin associates with Na(+)/H(+) exchanger type-1. FEBS Lett 2001;507:331–335. [PubMed: 11696366]

27. Zaun HC, Shrier A, Orlowski J. Calcineurin B homologous protein 3 promotes the biosynthetic maturation, cell surface stability, and optimal transport of the Na+/H+ exchanger NHE1 isoform. J Biol Chem 2008;283:12456–12467. [PubMed: 18321853]

28. Li X, Ding J, Liu Y, Brix BJ, Fliegel L. Functional analysis of acidic amino acids in the cytosolic tail of the Na+/H+ exchanger. Biochemistry 2004;43:16477–16486. [PubMed: 15610042]

29. Oh H, Siano B, Diamond S. Neutrophil isolation protocol. J Vis Exp. 2008

30. Owen PJ, Johnson GD, Lord JM. Protein kinase C-delta associates with vimentin intermediate filaments in differentiated HL60 cells. Exp Cell Res 1996;225:366–373. [PubMed: 8660925]

31. Sundstrom C, Nilsson K. Establishment and characterization of a human histiocytic lymphoma cell line (U-937). Int J Cancer 1976;17:565–577. [PubMed: 178611]

32. Favata MF, Horiuchi KY, Manos EJ, Daulerio AJ, Stradley DA, Feeser WS, Van Dyk DE, Pitts WJ, Earl RA, Hobbs F, Copeland RA, Magolda RL, Scherle PA, Trzaskos JM. Identification of a novel inhibitor of mitogen-activated protein kinase kinase. J Biol Chem 1998;273:18623–18632. [PubMed: 9660836]

33. Alessi DR, Cuenda A, Cohen P, Dudley DT, Saltiel AR. PD 098059 is a specific inhibitor of the activation of mitogen-activated protein kinase kinase in vitro and in vivo. J Biol Chem 1995;270:27489–27494. [PubMed: 7499206]

34. Dudley DT, Pang L, Decker SJ, Bridges AJ, Saltiel AR. A synthetic inhibitor of the mitogen-activated protein kinase cascade. Proc Natl Acad Sci U S A 1995;92:7686–7689. [PubMed: 7644477]

35. Dimberg A, Bahram F, Karlberg I, Larsson LG, Nilsson K, Oberg F. Retinoic acid-induced cell cycle arrest of human myeloid cell lines is associated with sequential down-regulation of c-Myc and cyclin E and posttranscriptional upregulation of p27(Kip1). Blood 2002;99:2199–2206. [PubMed: 11877298]
36. Kontani K, Nishina H, Ohoka Y, Takahashi K, Katada T. NAD glycohydrolase specifically induced by retinoic acid in human leukemic HL-60 cells. Identification of the NAD glycohydrolase as leukocyte cell surface antigen CD38. J Biol Chem 1993;268:16895–16898. [PubMed: 8394323]
37. Munshi CB, Graeff R, Lee HC. Evidence for a causal role of CD38 expression in granulocytic differentiation of human HL-60 cells. J Biol Chem 2002;277:49453–49458. [PubMed: 12386160]
38. Drach J, McQueen T, Engel H, Andreeff M, Robertson KA, Collins SJ, Malavasi F, Mehta K. Retinoic acid-induced expression of CD38 antigen in myeloid cells is mediated through retinoic acid receptor-alpha. Cancer Res 1994;54:1746–1752. [PubMed: 7511050]
39. Dominguez-Sola D, Ying CY, Grandori C, Ruggiero L, Chen B, Li M, Galloway DA, Gu W, Gautier J, Dalla-Favera R. Non-transcriptional control of DNA replication by c-Myc. Nature 2007;448:445–451. [PubMed: 17597761]
40. Fernandez PC, Frank SR, Wang L, Schroeder M, Liu S, Greene J, Cocito A, Amati B. Genomic targets of the human c-Myc protein. Genes Dev 2003;17:1115–1129. [PubMed: 12695333]
41. Grandori C, Cowley SM, James LP, Eisenman RN. The Myc/Max/Mad network and the transcriptional control of cell behavior. Annu Rev Cell Dev Biol 2000;16:653–699. [PubMed: 11031250]
42. Patel JH, Loboda AP, Showe MK, Showe LC, McMahon SB. Analysis of genomic targets reveals complex functions of MYC. Nat Rev Cancer 2004;4:562–568. [PubMed: 15229481]
43. Bentley DL, Groudine M. A block to elongation is largely responsible for decreased transcription of c-myc in differentiated HL60 cells. Nature 1986;321:702–706. [PubMed: 3520340]
44. Grosso LE, Pitot HC. Transcriptional regulation of c-myc during chemically induced differentiation of HL-60 cultures. Cancer Res 1985;45:847–850. [PubMed: 2578312]
45. Johansen LM, Iwama A, Lodie TA, Sasaki K, Felsher DW, Golub TR, Tenen DG. c-Myc is a critical target for c/EBPalpha in granulopoiesis. Mol Cell Biol 2001;21:3789–3806. [PubMed: 11340171]
46. Yen A, Roberson MS, Varvayanis S. Retinoic acid selectively activates the ERK2 but not JNK/SAPK or p38 MAP kinases when inducing myeloid differentiation. In Vitro Cell Dev Biol Anim 1999;35:527–532. [PubMed: 10548434]
47. Ueda Y, Hirai S, Osada S, Suzuki A, Mizuno K, Ohno S. Protein kinase C activates the MEK-ERK pathway in a manner independent of Ras and dependent on Raf. J Biol Chem 1996;271:23512–23519. [PubMed: 8798560]
48. Tonetti DA, Henning-Chubb C, Yamanishi DT, Huberman E. Protein kinase C-beta is required for macrophage differentiation of human HL-60 leukemia cells. J Biol Chem 1994;269:23230–23235. [PubMed: 8083228]
49. O'Brian CA, Ward NE. Biology of the protein kinase C family. Cancer Metastasis Rev 1989;8:199–214. [PubMed: 2697470]
50. Das D, Pintucci G, Stern A. MAPK-dependent expression of p21(WAF) and p27(kip1) in PMA-induced differentiation of HL60 cells. FEBS Lett 2000;472:50–52. [PubMed: 10781803]
51. Meighan-Mantha RL, Wellstein A, Riegel AT. Differential regulation of extracellular signal-regulated kinase 1 and 2 activity during 12-O-tetradecanoylphorbol 13-acetate-induced differentiation of HL-60 cells. Exp Cell Res 1997;234:321–328. [PubMed: 9260900]
52. Chambon P. A decade of molecular biology of retinoic acid receptors. FASEB J 1996;10:940–954. [PubMed: 8801176]
53. Kastner P, Mark M, Chambon P. Nonsteroid nuclear receptors: what are genetic studies telling us about their role in real life? Cell 1995;83:859–869. [PubMed: 8521510]
54. Mangelsdorf DJ, Evans RM. The RXR heterodimers and orphan receptors. Cell 1995;83:841–850. [PubMed: 8521508]
55. Takahashi N, Breitman TR. Retinoylation of HL-60 proteins. Comparison to labeling by palmitic and myristic acids. J Biol Chem 1990;265:19158–19162. [PubMed: 2229069]

56. Fanjul A, Dawson MI, Hobbs PD, Jong L, Cameron JF, Harlev E, Graupner G, Lu XP, Pfahl M. A new class of retinoids with selective inhibition of AP-1 inhibits proliferation. Nature 1994;372:107–111. [PubMed: 7969403]

57. Kamei Y, Xu L, Heinzel T, Torchia J, Kurokawa R, Gloss B, Lin SC, Heyman RA, Rose DW, Glass CK, Rosenfeld MG. A CBP integrator complex mediates transcriptional activation and AP-1 inhibition by nuclear receptors. Cell 1996;85:403–414. [PubMed: 8616895]

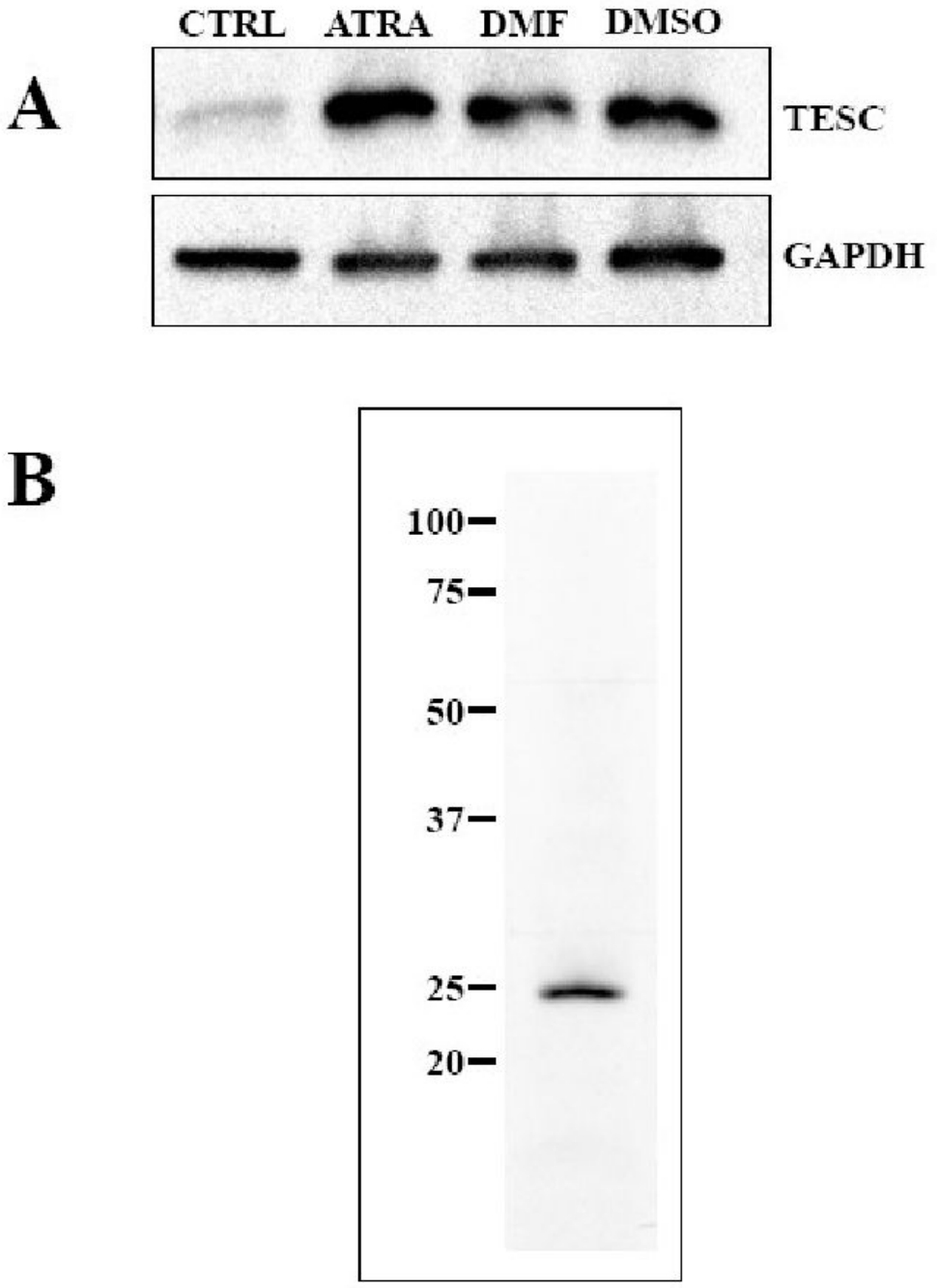

Figure 1. Expression of tescalcin during granulocytic differentiation of HL-60 cells and in primary granulocytes

(A) HL-60 cells were cultured as described in Materials and Methods and treated for 5 days either with 1mM ATRA, 1.25% DMSO, 80 mM DMF to induce granulocytic differentiation or with 0.1% DMSO vehicle as a control (CTRL). Cells were harvested in a lysis buffer on ice and approximately 15 μg of total proteins were analyzed by Western blot with antibody against tescalcin. Nitrocellulose membranes were then stripped and re-probed with antibody against GAPDH as a loading control. **(B)** Human polymorphonuclear granulocytes were isolated from peripheral blood as described in Material and Methods. Total protein extract (15 μg) was

resolved on 10-20% gradient polyacrylamide gel (Novex) and analyzed by Western blot with antibody against tescalcin.

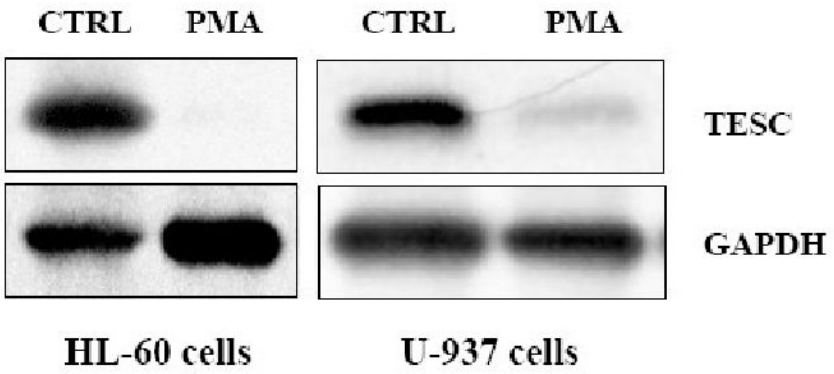

Figure 2. Downregulation of tescalcin during monocyte/macrophage differentiation of HL-60 and U-937 cells

To induce monocyte/macrophage-like differentiation, HL-60 or U-937 cells were treated for 5 days with 16 nM PMA or with 0.1% DMSO vehicle (CTRL). The level of tescalcin in total cell lysates was analyzed by Western blot with antibody against tescalcin. Antibody against GAPDH was used to show equal protein loading. Approximately 60 μg of total protein was loaded.

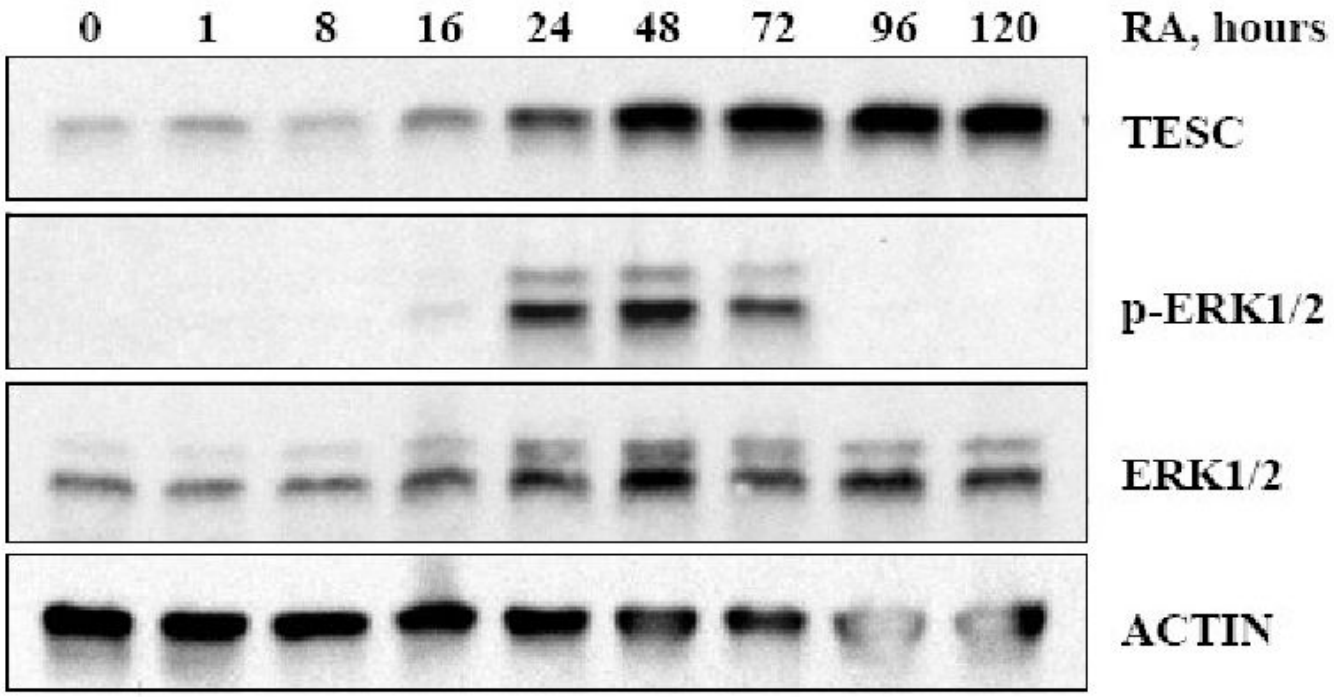

Figure 3. Time-course of tescalcin upregulation and activation of ERK during granulocytic differentiation of HL-60 cells

Exponentially growing HL-60 cells were stimulated with 1μM ATRA to induce granulocytic differentiation. Samples were collected at indicated times and analyzed by immunoblotting with antibodies against tescalcin, total p42/44 (ERK1/2) and phosphorylated p42/44 (p-ERK1/2). Antibody against β-actin was used to demonstrate equal protein loading.

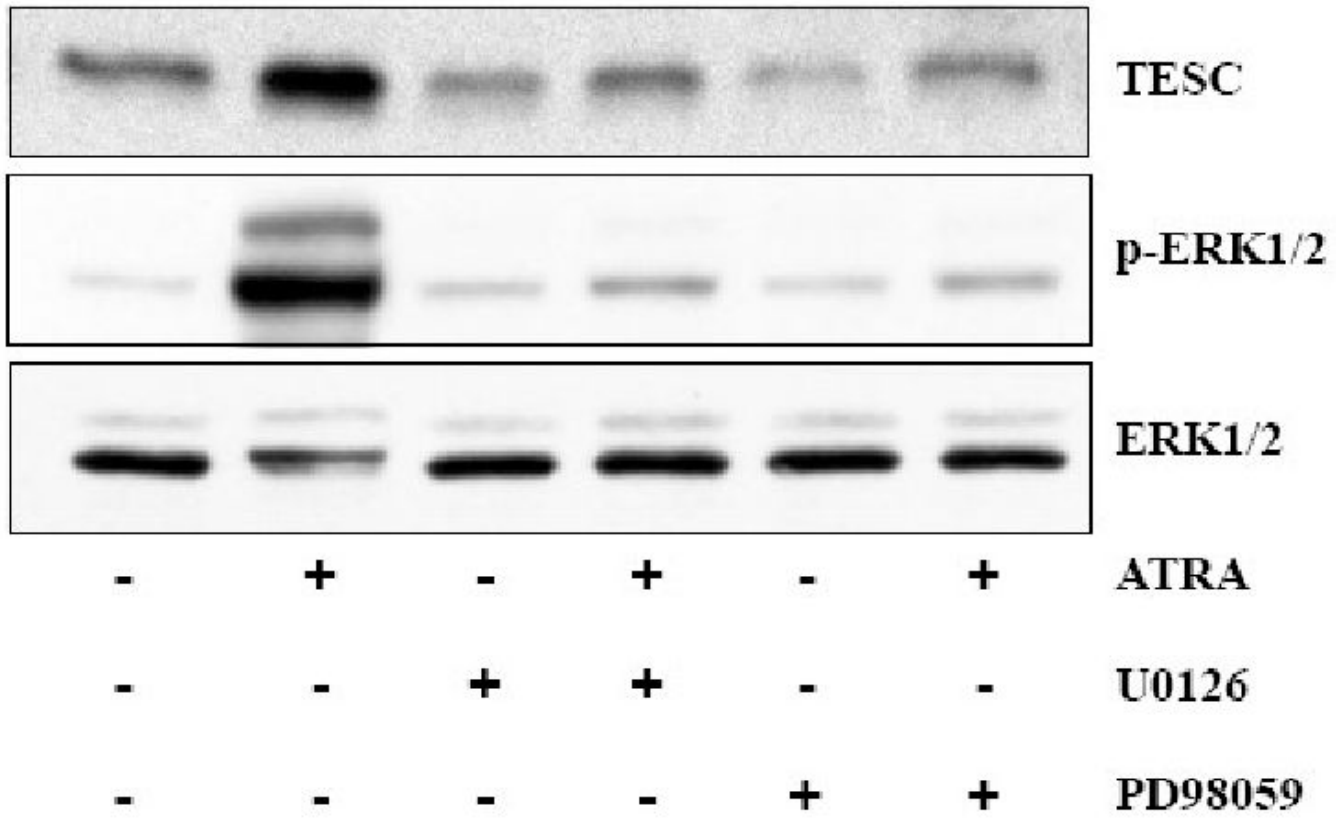

Figure 4. ATRA-induced tescalcin upregulation requires activation of ERK

MEK inhibitors U0126 (final concentration in the medium 4 μM) or PD98059 (10 μM) were added to cultured HL-60 cells for 30 min prior to induction of granulocytic differentiation with ATRA. Cells were harvested 72 h. later, lysed on ice and analyzed by immunoblotting with antibodies against tescalcin, phosphorylated p42/44 (p-ERK1/2) and total p42/44 (ERK1/2).

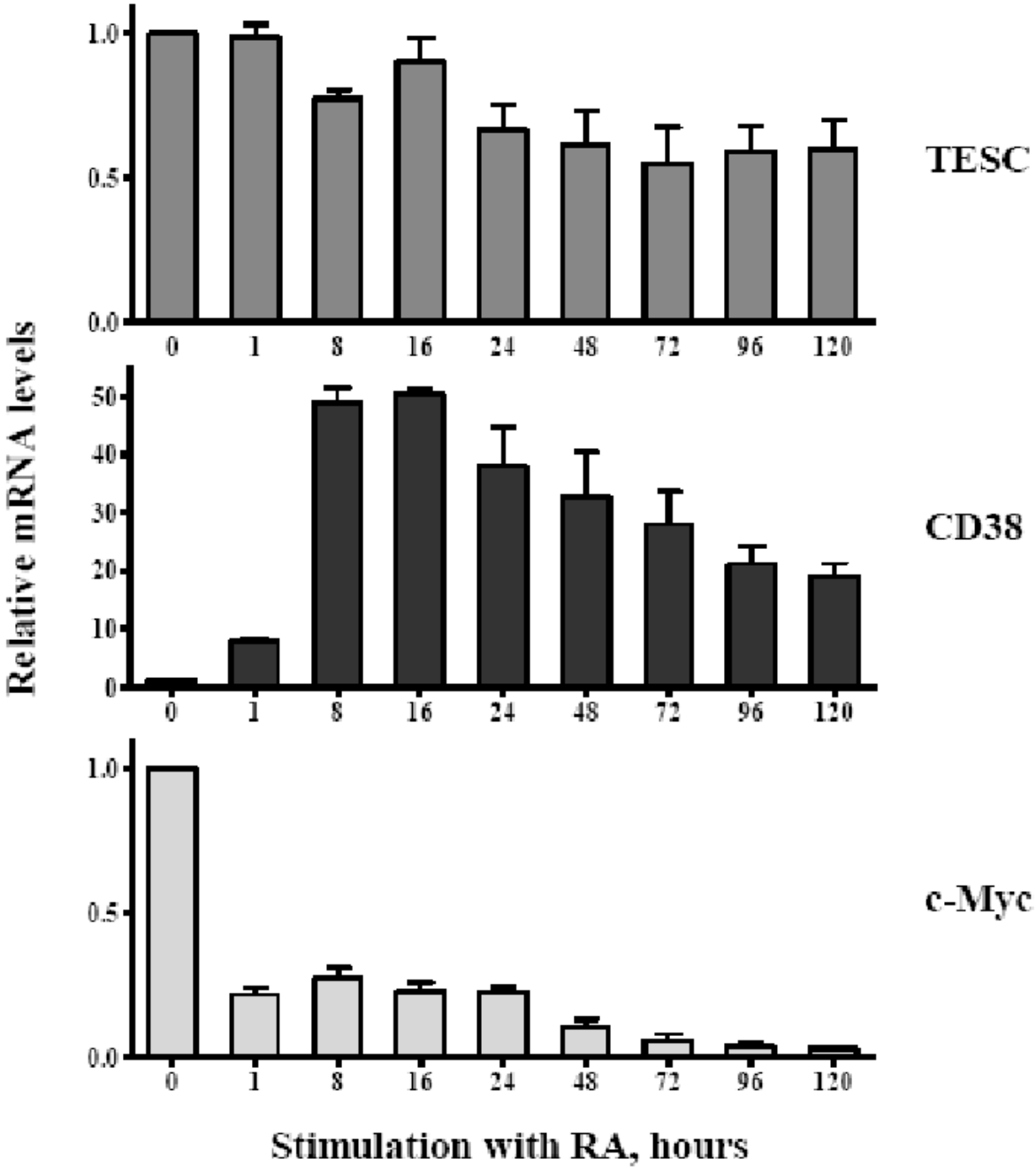

Figure 5. Tescalcin gene expression in HL-60 cells is not affected by ATRA

Exponentially growing cells were treated with 1μM ATRA to initiate granulocytic differentiation. At the indicated time points cells were collected and total RNA was purified using RNeasy kit (Qiagen). Total RNA was transcribed with High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). The relative expression levels of mRNAs encoding tescalcin, CD38, and c-Myc were analyzed by RQ-PCR and specific primers and probes (Taqman, Applied Biosystems). Reactions were run in triplicates on 7900HT Fast Real Time PCR system (Applied Biosystems) and normalized to the endogenous control 18S ribosomal RNA. Data are expressed as fold change relative to the gene expression levels at time 0 (mean ±sd).

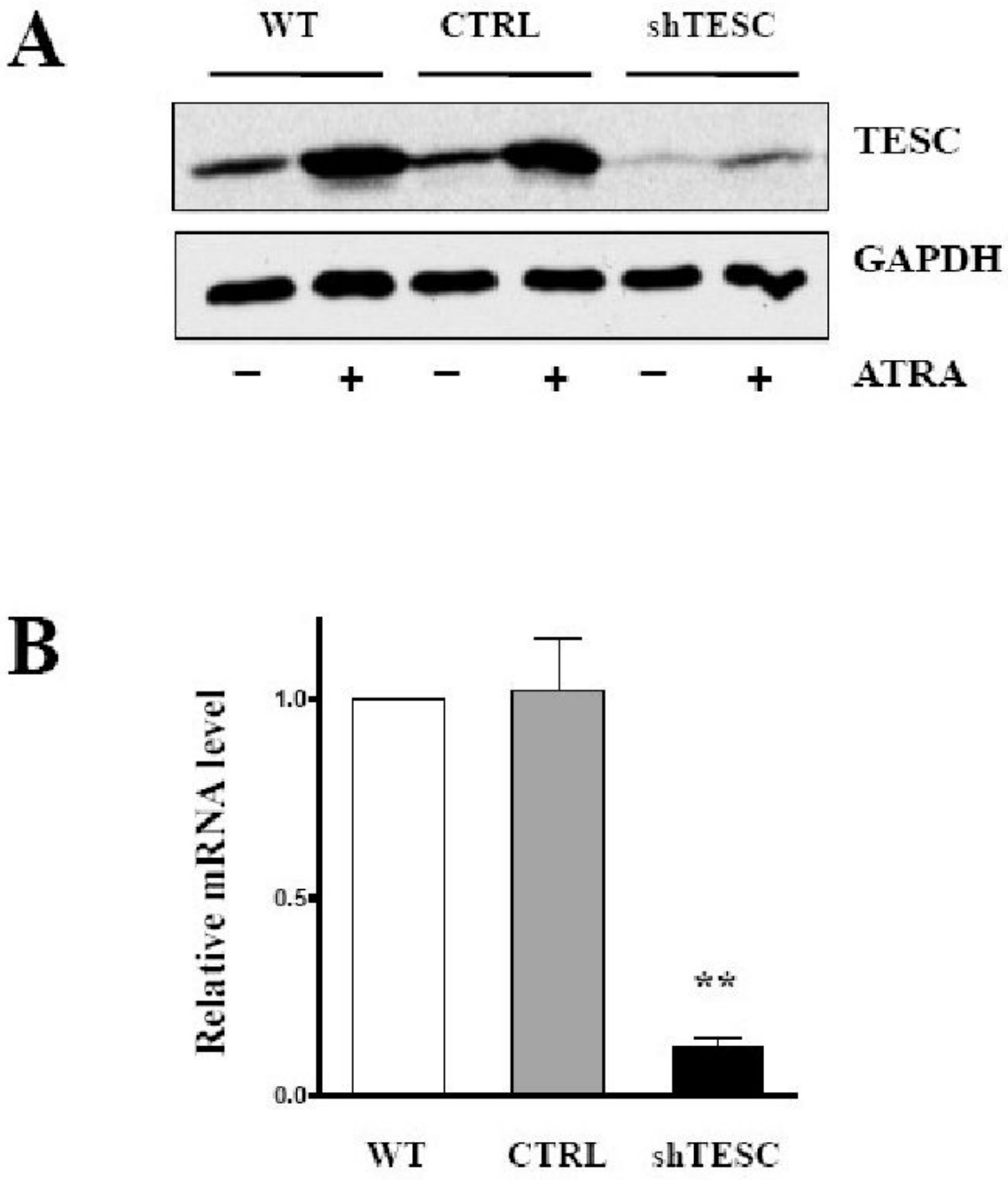

Figure 6. shRNA-mediated knockdown of tescalcin in HL-60 cells

(A) HL-60 cells were electroporated with plasmids encoding scrambled (CTRL) and Tescalcin-specific shRNA (shTESC), then selected for neomycin resistance. Expression of tescalcin in non-induced and ATRA-induced cells was detected by Western blot and compared to the wild-type HL-60. GAPDH is shown as loading control. **(B)** Total RNA was extracted from cells expressing either scrambled or tescalcin-specific shRNA and analyzed by RQ-PCR using specific primers and probes. Reactions were run in triplicates on 7900HT Fast Real Time PCR system (Applied Biosystems) and normalized to the endogenous control 18S ribosomal RNA. Data are expressed relative to wild-type HL-60 (mean±sd). Statistical analysis on results was

performed using paired, two-tailed Student's *t* test and marked with a double asterisk if $p < 0.01$.

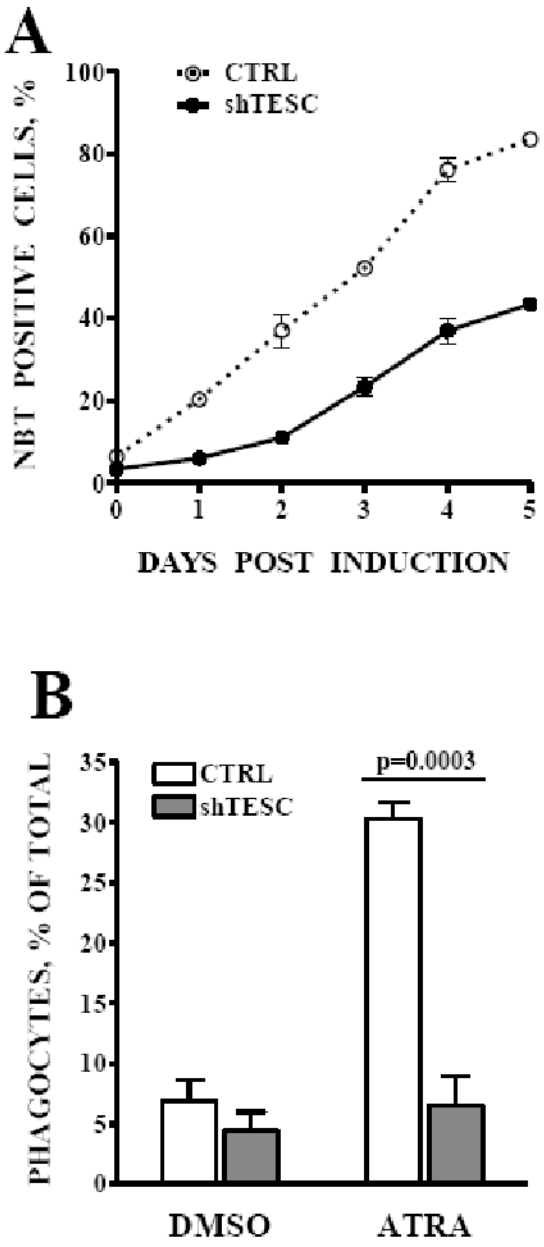

Figure 7. Tescalcin knockdown inhibits ATRA-induced granulocytic differentiation and maturation of HL-60 cells

Granulocytic differentiation was induced in HL-60 cells expressing scrambled or tescalcin-specific shRNA. **(A)** To measure the generation of superoxide, cells were collected at indicated times, washed with PBS, and incubated with 0.1% NBT as described in Material and Methods. The percentage of cells containing intracellular blue-black formazan deposits was then determined on Wright-Giemsa stained slide preparations. At least 400 cells were analyzed in each time point. **(B)** HL-60 cells were cultured with 1 μM ATRA or 0.1% DMSO (CTRL) for 72 hours. To study phagocytic activity, the cells were washed and incubated in fresh culture media with 0.025% of 1 μm fluorescent carboxylate-modified microspheres (Invitrogen). The fluorescence uptake was analyzed by flow cytometry as described under Material and Methods, with a minimum of 10,000 events acquired per sample. The number of cells ingested the fluorescent microspheres is expressed as % of total cells gated. Assays were performed in

triplicates. Bar graph represents results of three independent experiments (mean±sd). Statistical analysis was performed using paired, two-tailed Student's *t* test.

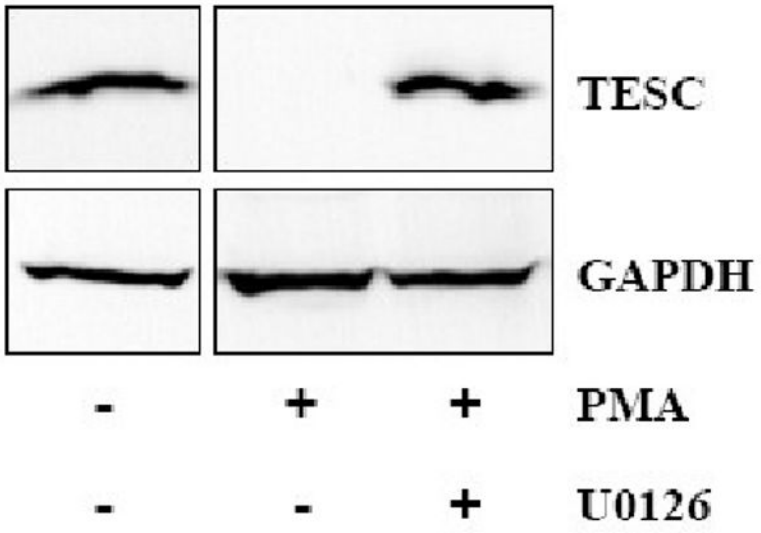

Figure 8. Downregulation of tescalcin by PMA requires activation of ERK

HL-60 cells were cultured for 1 hour in the absence or presence of 4 μM U0126 MEK inhibitor prior to treatment with 16 nM PMA. Total protein extracts were prepared from cells harvested 72 hours later, analyzed by immunoblotting with tescalcin antibody, and compared to untreated HL-60. Antibody against GAPDH was used to show equal protein loading.

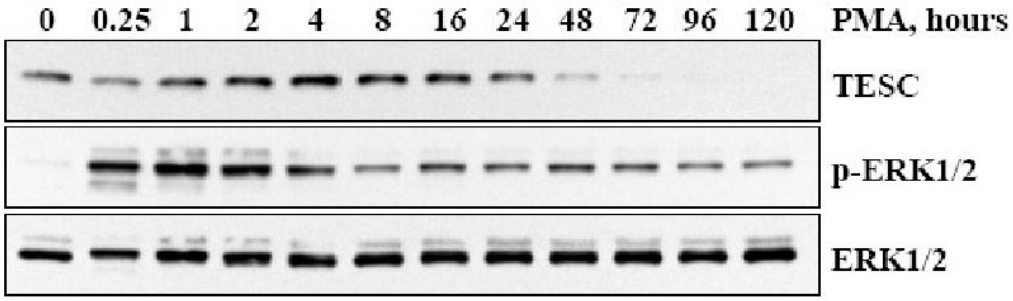

Figure 9. Kinetics of tescalcin downregulation and activation of ERK during PMA-induced macrophage-like differentiation

Cultured HL-60 cells were treated with 16 nM PMA to stimulate macrophage-like differentiation. Cells were harvested at the indicated times and total protein lysates were subjected to PAGE. Resolved proteins were analyzed by immunoblotting with antibodies against tescalcin, total p42/44 (ERK1/2) and phospho-p42/44 (p-ERK1/2).

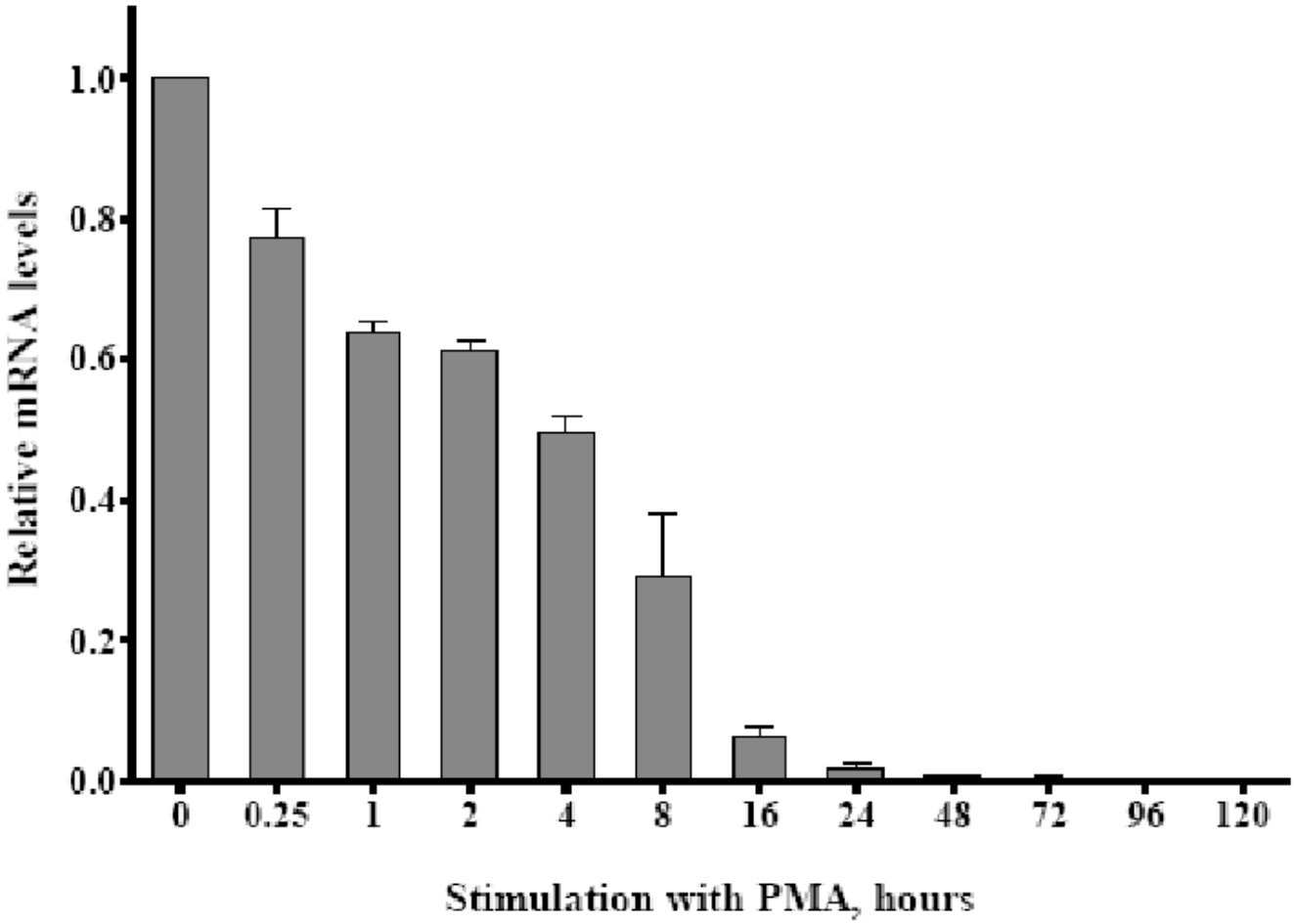

Figure 10. PMA-mediated downregulation of tescalcin gene expression

To initiate macrophage-like differentiation, HL-60 cells were treated with 16 nM PMA. Total RNA was harvested from cells at the indicated time points, transcribed with High Capacity cDNA Reverse Transcription Kit and analyzed by RQ-PCR using tescalcin-specific primers and probes (Taqman, Applied Biosystems). Reactions were run in triplicates on 7900HT Fast Real Time PCR system (Applied Biosystems) and normalized to 18S rRNA. Data are expressed as fold change relative to the gene expression levels at time 0 (mean±sd).