In Vitro Cell.Dev.Biol.—Animal (2009) 45:264–274
DOI 10.1007/s11626-008-9163-4

Granulocytic differentiation of HL-60 promyelocytic leukemia cells is associated with increased expression of Cul5

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Received: 22 May 2008 / Accepted: 3 December 2008 / Published online: 1 January 2009 / Editor: J. Denry Sato

Abstract The human HL-60 promyelocytic leukemia cell line has been widely used as a model for studying granulocytic differentiation. All-*trans* retinoic acid (ATRA) treatment of HL-60 cells promotes granulocytic differentiation and is effective as differentiation therapy for patients with acute promyelocytic leukemia. The identification of genes that are transcriptionally regulated by ATRA has provided insight into granulocytic differentiation and differentiation therapy. The *Asb-2* (ankyrin repeat SOCS box 2) gene has previously been identified as a transcriptional target in ATRA-treated HL-60 cells. The ASB-2 protein forms an E3 ubiquitin ligase complex with the proteins, Cul5, regulator of cullin 2 (ROC2), and elongin B and C. The purpose of this study was to determine if there is increased expression of Cul5 during granulocytic differentiation of HL-60 cells. To induce granulocytic differentiation, HL-60 cells were treated for 5 d with ATRA and differentiation was confirmed by examining superoxide anion production, nuclear morphology, and changes in the expression of CD11b, CD13, and CD15. Quantitative real-time RT-PCR was used to measure Cul5 mRNA expression and also the expression of other components of the E3 ubiquitin ligase (ASB-2, ROC2, elongin B and C). Granulocytic differentiation of HL-60 cells was associated with a 1.6-, 1.7-, and 23-fold statistically significant ($P \leq 0.05$) increase in mRNA expression for Cul5, ROC2, and ASB-2, respectively. No significant change was found in elongin B and C mRNA expression. Using Western blot analysis, the expression of Cul5 protein was increased 6.5-fold with granulocytic differentiation of the HL-60 cells. Increased expression of multiple components of the Cul5-containing E3 ubiquitin ligase complex with ATRA treatment of HL-60 cells indicates that this complex may play an important role in granulocytic differentiation.

Keywords HL-60 · Granulocytic differentiation · All-*trans* retinoic acid · Cul5 · ROC2 · ASB-2 · Elongin B/C

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Introduction

The human myeloid HL-60 cell line was established from a patient with acute promyelocytic leukemia (APL) (Collins et al. 1977; Gallagher et al. 1979) and has been widely used as a model for studying granulocytic and macrophage-like differentiation (Harris and Ralph 1985; Tsiftsoglou and Robinson 1985; Collins 1987; Birnie 1988). Retinoid treatment of HL-60 cells promotes granulocytic differentiation (Breitman et al. 1980) and all-*trans* retinoic acid (ATRA) is effective as differentiation therapy for patients with APL (Huang et al. 1988; Castaigne et al. 1990; Warrell et al. 1991). The mechanism of ATRA-induced granulocytic differentiation involves the binding of ATRA to retinoic acid receptor alpha (RARα), which then forms a heterodimer with the retinoid X receptor (RXR). The RAR/RXR complex then binds to a DNA sequence called the retinoic acid response element (RARE) in the promoter region of target genes to regulate

transcription (Giguere 1994). The identification of genes that are induced with ATRA treatment of HL-60 cells should provide insight into granulocytic differentiation and the response of APL cells to differentiation drug therapy.

A gene that has been shown to be induced during ATRA-mediated differentiation of HL-60 cells is the *Asb-2* (ankyrin repeat SOCS box 2) gene (Kohroki et al. 2001; Guibal et al. 2002). The ASB-2 protein is a member of the suppressor of cytokine signaling (SOCS) protein family and is characterized by protein motifs called ankyrin repeats and the SOCS box (Hilton et al. 1998; Kile et al. 2000). Some members of the SOCS box family of proteins have been shown to act as negative regulators of cytokine signaling, however, the cellular effects of ASB-2 are not as well understood (Krebs and Hilton 2000). ASB-2 expression is induced by ATRA in APL cell lines and over-expression of ASB-2 promotes growth inhibition and chromatin condensation, which are characteristics of ATRA-differentiated APL cells (Kohroki et al. 2001; Guibal et al. 2002). Studies have shown that ASB-2 interacts with a scaffolding protein (Cul5/cullin-5), a RING finger protein (ROC/Rbx), and an adapter protein complex (elongin B/C) to form an ECS (elongin B/C-Cul5-SOCS box) E3 ubiquitin ligase complex (Kamura et al. 1998; Kile et al 2002; Heuze et al. 2005; Kohroki et al. 2005). These ECS E3 ubiquitin ligases function to bring together an E2 ubiquitin-conjugating enzyme and a target protein which is specifically recognized by the SOCS-box protein. Once a target protein is recognized by this ECS E3 ubiquitin ligase complex, the E2 enzyme polyubiquitinates the target, leading to the degradation of the target protein by the 26S proteasome (Hershko and Ciechanover 1998; Deshaies 1999; Willems et al. 2004).

Since *Asb-2* has been identified as one of the genes transcriptionally up-regulated by ATRA, we investigated the possibility that other components of the ECS E3 ubiquitin ligase would also be up-regulated during ATRA-mediated granulocytic differentiation of HL-60 cells. The expression of Cul5, regulator of cullin 2 (ROC2), and ASB-2 mRNA was significantly increased with ATRA-mediated granulocytic differentiation, while no significant change was found in elongin B and C mRNA expression. The expression of Cul5 protein was also significantly increased with granulocytic differentiation of the HL-60 cells. Studying the expression and function of genes that are up-regulated during ATRA-induced differentiation of HL-60 cells may provide insight into granulocytic differentiation and differentiation therapy.

Materials and Methods

Cell culture. The HL-60 human promyelocytic leukemia cell line (Collins et al. 1977; Gallagher et al. 1979) was directly obtained from the American Type Culture Collection (ATCC®, Manassas, VA) and was cultured in Iscove's Modified Dulbecco's medium+GlutaMAX™ (Invitrogen, Grand Island, NY) supplemented with 10% heat-inactivated fetal bovine serum (Hyclone, Logan, UT) and penicillin (100 units/mL)-streptomycin (100 μg/mL) (Invitrogen, Grand Island, NY). The cells were cultured as recommended by the ATCC® and maintained in a humidified incubator at 37°C with 5% CO_2–95% air. Cells used for experiments tested negative for mycoplasma infection using the MycoAlert® Mycoplasma detection kit (Lonza, Rockland, ME). For experiments, cell counts were performed using a hemacytometer and trypan blue (Sigma, St. Louis, MO) and cells were seeded into 75-cm^2 flasks at a concentration of 1×10^5 cells/mL. One day after seeding, cells were treated for 5 d with a final concentration of 1 μM ATRA (Sigma) to induce granulocytic differentiation or ethanol (AAPER Alcohol and Chemical Co., Shelbyville, KY) as the vehicle-control. A 10-mM ATRA stock solution was prepared in ethanol, aliquoted, stored at −20°C, and a fresh aliquot was used for each experiment. Three independent experiments were performed with triplicate vehicle-control flasks and triplicate ATRA-treated flasks for verification of differentiation and the real-time reverse transcription polymerase chain reaction (RT-PCR) experiments. For the Western blot experiments, six vehicle control and six ATRA-treated flasks were used.

Discontinuous superoxide anion assay. Phorbol 12-myrisate 13-acetate (PMA)-stimulated and spontaneous superoxide anion (O_2^-) generation was determined in ATRA-treated or vehicle-control HL-60 cells by measuring ferricytochrome C (FCC) reduction in the presence or absence of superoxide dismutase (SOD) as previously described (Mayer et al. 1995, 1996). Stock solutions of SOD (3,500 units/mL; Sigma, St. Louis, MO) and FCC (6.2 mg/mL; Sigma) were prepared using Hank's balanced salt solution (HBSS; Invitrogen) and stored at −80°C. The PMA was obtained from Sigma (St. Louis, MO) and an 8.02-mM stock solution was prepared using dimethyl sulfoxide (Sigma) and stored at −80°C. Assays were performed in 24-well plates with 5×10^5 cells per well in a final reaction volume of 1.0 mL. To measure spontaneous O_2^- generation in the ATRA-treated and vehicle-control cells, each well received 50 μM FCC with or without the addition of SOD (350 units). To measure PMA-stimulated O_2^- generation in the ATRA-treated and vehicle-control cells, each well received 50 μM FCC and 1 μM PMA with or without the addition of SOD (350 units). After 90 min of incubation at 37°C, the samples were spun at 300×*g* for 10 min at 4°C. The supernatants were then transferred to cuvettes and the absorbance was read at 550 nm using a Beckmann Coulter DU800 spectrophotometer (Fullerton, CA). The data for

O_2^- generation are expressed as nmoles O_2^-/500,000 cells/90 min and was calculated using the differences for FCC reduction in samples with and without SOD and an extinction coefficient of 21.0×10^3 M^{-1} cm^{-1}. Statistical analysis was performed using GraphPad Prism® version 4.0 software (GrapPad Software, San Diego, CA) and two-way ANOVA followed by the Bonferroni post-hoc test ($P\leq0.05$).

Wright–Giemsa staining. To assess the nuclear morphology changes in the ATRA-treated HL-60 cells, Wright–Giemsa staining was performed. Briefly, approximately 5×10^4 ATRA-treated or vehicle-control cells were resuspended in 0.5 mL of Iscove's Modified Dulbecco's medium+GlutaMAX™ and centrifuged for 10 min at 500×*g* onto glass microscope slides, using a Shandon Cytospin 3 (Shandon, Pittsburgh, PA). The slides were allowed to air dry at room temperature for approximately 1 h. Then each slide was stained for 30 s using Accustain® Wright–Giemsa stain (Sigma) and then immediately placed in deionized water for approximately 10 min. Finally, the slides were rinsed briefly in deionized water and allowed to air dry at room temperature. Slides were visualized using light microscopy and the nuclear morphology was classified as ovoid, indented, lobulated, or multilobed, as previously described by Olins and Olins (2004), from a total of 1,877 vehicle-control cells and 1,281 ATRA-treated cells.

Flow cytometry analysis. Flow cytometry was used to monitor changes in the expression of cell surface antigens during the differentiation of ATRA-treated HL-60 cells according to the protocol of Givan (2000). Briefly, ATRA-treated or vehicle-control cells were washed in stain/wash buffer (S/W) which consisted of PBS (Invitrogen) with 0.1% azide (Sigma) and 1.0% bovine serum albumin (BSA; Sigma). Cells were then resuspended in S/W buffer at 5×10^7 cells/mL. The cell suspension was mixed with an equal volume of blocking buffer, which consisted of 1.2% human γ-globulin (Sigma) and S/W buffer. Then 40 µl aliquots of the cell suspension with blocking buffer were added to tubes containing 20 µl of each of the FITC conjugated primary monoclonal antibodies at the concentration recommended by the manufacturer (eBioscience, San Diego, CA; Biolegend, San Diego, CA). The antibodies used and the corresponding isotype controls were as follows: anti-mouse CD11b and rat IgG2b isotype control, anti-human CD13, and mouse IgG1 κ isotype control, and anti-human CD15 and mouse IgM κ isotype control. After an incubation period of 30 min in the dark at 4°C, the samples were washed a total of three times in S/W buffer, then resuspended in 150–250 µl of S/W buffer and an equal volume of cold 2.0% *p*-formaldehyde (Sigma) in PBS. Replicates for each ATRA-treated or vehicle-control sample were processed as described above and stored at 4°C in the dark. Approximately 20,000 events per sample were analyzed using a Coulter® EPICS XL™ Flow Cytometer (Beckman Coulter) and the FCS Express Software (De Novo, Los Angeles, CA) at the Robert H. Lurie Comprehensive Cancer Center Flow Cytometry Core Facility at Northwestern University (Chicago, IL).

Reverse transcription (RT) and real-time PCR. Total RNA was isolated using TRI REAGENT® (Molecular Research, Cincinnati, OH) according to the protocol from the manufacturer which is based on the method of Chomczynski and Sacchi (1987). Integrity of the RNA was assessed by visualization of ethidium bromide stained 18S and 28S ribosomal RNA bands and by determining the 260/280 nm ratio. Single-stranded cDNA synthesis was performed using the high-capacity cDNA RT Kit (Applied Biosystems, Foster City, CA). The 20-µl reactions contained 2 µl of 10× RT buffer, 0.8 µl of 25× dNTP Mix (100 mM), 2 µl of 10× random primers, 1 µl of MultiScribe™ reverse transcriptase, and 14.2 µl of nuclease-free water. The RT was performed using a PTC 200 DNA Engine Peltier thermal cycler (MJ Research, Waltham, MA) and the following conditions: 25°C for 10 min, 37°C for 2 h, and 85°C for 5 s. An RNA dilution series (1:2) ranging from 25 ng to 1,600 ng using total RNA from non-treated HL-60 cells was used in the RT reactions for the validation experiments and generation of standard curves. For the ATRA-treated and vehicle-control samples, 100 ng of total RNA was used for each RT reaction. Real-time PCR was performed using Taqman® Assays and the 7300 Real-time PCR System from Applied Biosystems. The 25 µl reactions contained 12.5 µl of Taqman® Universal PCR Master Mix with no AmpErase® UNG, 8.75 µl of nuclease-free water, 2.5 µl of cDNA, and 1.25 µl of Taqman® Gene Expression Assay. The Taqman® Gene Expression Assays used for this study were glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (part# 4333764T), ASB-2 (ID# Hs00387867_m1), ROC2 (ID# Hs00762035_s1), Cul5 (ID# Hs00180143_m1), elongin B (ID# Hs00793006_m1), and elongin C (ID# Hs00855349_g1). The PCR cycling conditions were 10 min at 95°C (DNA polymerase activation), followed by 40 cycles of 15 s at 95°C (melt), and 1 min at 60°C (anneal/extend). For real-time PCR experiments, each plate contained three to five replicate wells per sample. The real-time PCR data were analyzed using 7300 Real-Time PCR System RQ study software version 1.4 (Applied Biosystems) to obtain a relative quantification value.

Validation experiments were performed to ensure that the gene specific assays and the GAPDH endogenous control assay had similar efficiencies. The validation process consisted of generating graphs of the ΔC_T values (Target C_T−GAPDH C_T) versus the log of the RNA input and the assays passed validation if the slope of the plot was

<0.1. Using this method, the Cul5, ROC2, and elongin B and C assays passed validation; however, ASB-2 failed the validation test (data not shown). As a result of the validation experiments, the $\Delta\Delta C_T$ method was used for relative quantification of Cul5, ROC2, and elongin B and C, while both the $\Delta\Delta C_T$ and the relative standard curve methods were used for ASB-2. The $\Delta\Delta C_T$ method uses the arithmetic formula $2^{-\Delta\Delta CT}$ to calculate the fold difference in gene expression between a test sample and a calibrator sample, where $\Delta\Delta C_T = \Delta C_T$ test sample (ATRA-treated) $-\Delta C_T$ calibrator sample (vehicle-control). With the relative standard curve method, ASB-2 and GAPDH mRNA expression were determined by extrapolating the amount of ATRA-treated and vehicle-control samples using a standard curve produced with RNA from untreated HL-60 cells. The sample amounts were normalized by dividing the extrapolated sample quantity by the extrapolated GAPDH quantity, and then the fold difference was calculated by dividing the normalized test (ATRA-treated) sample value by the normalized calibrator (vehicle-control) sample value.

Statistical analysis was performed on the ΔC_T values (Target C_T − GAPDH C_T) for vehicle-control versus ATRA-treated samples using two-tailed Student's t test ($P \leq 0.05$) as previously described (Yuan et al. 2006).

SDS-PAGE and Western blot analysis. Cellular protein was isolated using the M-PER® (Pierce, Rockford, IL) mammalian protein extraction reagent supplemented with a complete™ mini protease inhibitor cocktail tablet (Roche Molecular Biochemicals, Indianapolis, IN) as previously described (Fay et al. 2003). To facilitate equal loading between samples, protein concentrations were determined using the BCA™ kit (Pierce). For each sample ~50µg of protein was diluted in 6× SDS sample buffer and boiled for 5 min. SDS-PAGE was performed using 15% acrylamide gels (BIO-RAD, Hercules, CA) (Laemmli 1970), and proteins were transferred to PVDF membranes (BIO-RAD) overnight at 4°C. Membranes were blocked for 1 h at room temperature using Superblock® (Pierce) followed by incubation for 1 h at room temperature with a rabbit anti-Cul5 polyclonal antibody (Invitrogen) diluted (1:125) in antibody dilution buffer (10% Superblock®/90% 10 mM Tris, pH 7.5, 100 mM NaCl, and 0.1% Tween® 20). Following primary antibody, the membranes were washed (6×5 min) in wash buffer (10 mM Tris, pH 7.5, 100 mM NaCl, and 0.1% Tween® 20), and incubated with a 1:10,000 dilution of immunopure® peroxidase conjugated goat anti-Rabbit secondary antibody (Pierce) for 1 h at room temperature. Membranes were washed as previously described and incubated with the Supersignal® west pico chemiluminescent substrate (Pierce) for 5 min. A Kodak Gel Logic 1500 imaging system (Kodak, Rochester, NY) was used for visualization of the Western blots using 10 min exposure times. The membranes were then stripped using the Restore™ Western blot stripping buffer (Pierce) and were blocked as previously described and reprobed with a rabbit anti-elongin C polyclonal antibody (BioLegend) at a dilution of 1:500. Analysis was performed using the Kodak molecular imaging software (version 4.0) and molecular weights were determined by comparison of experimental bands to Kaleidoscope prestained standards (BIO-RAD). A net intensity value for Cul5 and elongin C was obtained by drawing regions of interest of equal area around each band and subtracting a local background value. The data were normalized for loading differences by dividing the Cul5 net intensity value by the elongin C net intensity value. The data were obtained using six independent samples per group and statistical analysis was performed using a two-tailed Student's t test ($P \leq 0.05$).

Results

Assessment of ATRA-mediated granulocytic differentiation of HL-60 cells. To assess ATRA-mediated granulocytic differentiation of HL-60 cells, viable cell counts and percent viability were determined. As shown in Fig. 1*A*, the mean number of viable cells for the ATRA-treated cultures decreased 41% compared to the vehicle-control cells, while the percent viability of the ATRA-treated cultures (92.3%) and the vehicle-control cultures (96.5%) were similar (Fig. 1*B*).

Nuclear morphology analysis of Wright–Giemsa stained HL-60 cells after ATRA or vehicle treatment was used as another assessment of granulocytic differentiation. In accordance with a previous study (Olins and Olins 2004), four nuclear morphologies were identified: ovoid (Fig. 2*A*), indented (Fig. 2*B*), lobulated (Fig. 2*C*), and multilobed (Fig. 2*D*). The distinction between the lobulated and multilobed nuclei was based upon the number of lobes, with multilobed nuclei having ≥5 lobes. As shown in Fig. 2*E*, the mean percentage of cells with the ovoid morphology decreased from 94.37% in the vehicle-control group to 22.81% in the ATRA-treated group. In contrast, the percentage of cells with indented, lobulated, and multilobed morphologies increased from 3.69%, 1.87%, and 0.07% in the vehicle-control group to 56.06%, 17.21%, and 3.92% in the ATRA-treated group, respectively.

Superoxide anion generation was also used to evaluate granulocytic differentiation of the ATRA-treated HL-60 cells. Statistical analysis using a two-way ANOVA indicated a significant effect of PMA, a significant effect of ATRA, and a significant interaction. As shown in Fig. 3, post-hoc analysis indicated that the observed increase in PMA-stimulated O_2^- generation (26.48±1.64 nmol of O_2^-/

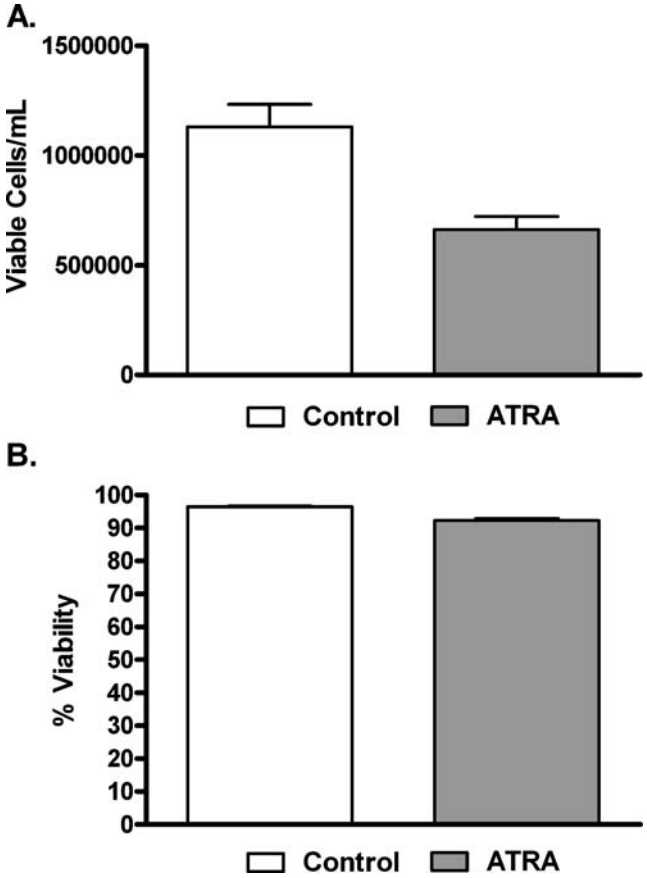

Figure 1. Viable cell counts and percent viability assessments for HL-60 cells treated with ATRA. Cells were treated for 5 d with 1 μM ATRA or vehicle-control and the viable cells/mL and % viability were determined using a hemacytometer and trypan-blue. *A* Viable cells/mL; *B* percent viability; the data are shown as the mean±SE; n=9 per group.

5×10^5 cells/90 min) was statistically significant ($P<0.001$) from spontaneous O_2^- release (3.23±1.44 nmol of O_2^-/5×10^5 cells/90 min) in the ATRA-treated cells. In contrast, spontaneous O_2^- generation (5.02±2.33 nmol of O_2^-/5×10^5 cells/90 min) was not significantly different ($P>0.05$) from PMA-stimulated O_2^- release (9.76±2.12 nmol of O_2^-/5×10^5 cells/90 min) in the vehicle-control cells.

Flow cytometry was also used to assess granulocytic differentiation of the ATRA-treated HL-60 cells by measuring the expression of the cell surface antigens CD11b, CD13, and CD15. Figure 4*A*, *C* show that the expression of CD11b and CD15 increased ~2.4-fold and ~2.2-fold in the ATRA-treated cells versus the vehicle-control cells, respectively. In contrast, CD13 expression decreased ~1.5-fold in the ATRA-treated cells versus the vehicle-control cells (Fig. 4*B*). The isotype control FITC-conjugated antibodies were used as negative controls, and staining of the vehicle-control and ATRA-treated cells was similar for all three isotype controls (Fig. 4*A*, *B*, *C*). The mean fluorescence values for the isotype controls were similar to unstained cells (data not shown).

Real-time RT-PCR analysis of ASB-2, Cul5, ROC2, and elongin B and C mRNA expression. Quantitative real-time RT-PCR was performed using Taqman® assays to measure ASB-2, Cul5, ROC2, and elongin B and C mRNA expression in ATRA-treated HL-60 cells versus vehicle-control cells. Using the relative standard curve method, there was a 23-fold statistically significant (two-tailed Student's t test, $P<0.05$) increase in ASB-2 mRNA expression in the ATRA-treated cells compared to the vehicle-control cells (Fig. 5*A*). Even though the *Asb-2* gene expression assay did not pass validation for performing the

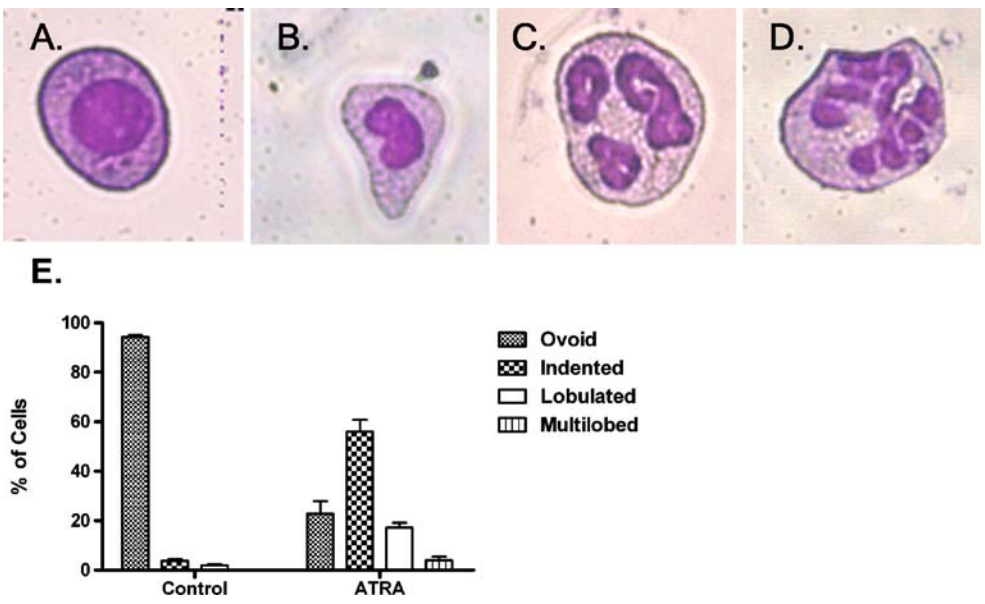

Figure 2. Nuclear morphology changes of HL-60 cells treated with ATRA. Cells were treated for 5 d with 1 μM ATRA or vehicle-control and the nuclear morphology of Wright–Giemsa stained was determined. *A* Ovoid, *B* indented, *C* lobulated, or *D* multilobed, *E* percent of cells in each category; the data are shown as the mean±SE; n=9 per group.

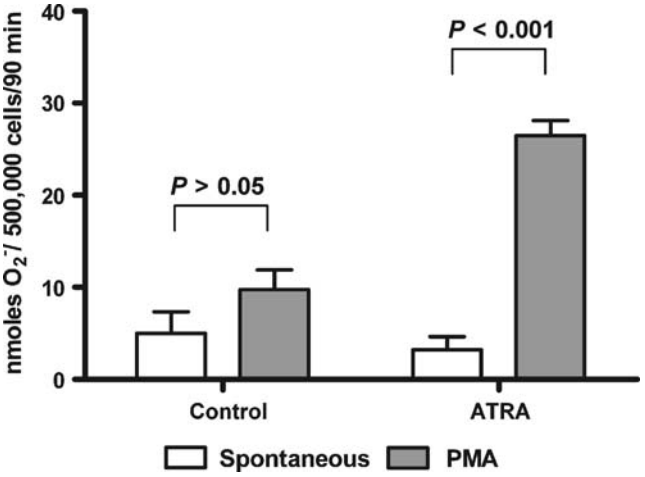

Figure 3. Spontaneous and PMA-stimulated O_2^- generation in the ATRA-treated and vehicle-control HL-60 cells. Cells were treated for 5 d with 1 μM ATRA or vehicle-control. The data for O_2^- generation are expressed as nmoles O_2^-/500,000 cells/90 min, and was calculated from absorbance readings at 550 nm using the differences for FCC reduction in samples with and without SOD and an extinction coefficient of 21.0×10^3 M^{-1} cm^{-1}. The data are shown as the mean± SE, n=9 per group. Statistical analysis was performed using two-way ANOVA followed by the Bonferroni post-hoc test ($P\leq0.05$).

$\Delta\Delta C_T$ method, the 24-fold increase in ASB-2 mRNA expression (*data not shown*) in ATRA-treated cells was similar to the results obtained using the relative standard curve method. There was a 1.6-fold statistically significant increase in Cul5 mRNA expression in the ATRA-treated cells compared to the vehicle-control cells (Fig. 5*B*). The expression of ROC2 mRNA was also significantly increased 1.7-fold in the ATRA-treated cells compared to the vehicle-control cells (Fig. 5*C*). In contrast, the expression levels of elongin B and elongin C mRNAs were not significantly different between the ATRA-treated and vehicle-control cells (Fig. 5*D*, *E*).

Western blot analysis of Cul5 protein expression. Western blot analysis was used to evaluate Cul5 protein expression in ATRA-treated HL-60 cells versus vehicle-control cells. Elongin C was used to normalize the blots for loading differences since no significant difference was observed in the expression of elongin C mRNA between the vehicle control and the ATRA treated HL-60 cells (Fig. 5*E*). As shown in Fig. 6*A*, a band of ~84 kDa was detected for Cul5 which is close to the predicted molecular weight of ~90 kDa and a band of the predicted molecular weight of ~15 kDa was detected for elongin C. There was a ~6.5-fold statistically significant increase in Cul5 protein expression in the ATRA-treated cells compared to the vehicle-control cells (Fig. 6*B*). Using commercially available antibodies from several companies and the methods described, we were unable to detect bands for ASB-2 and ROC2 of the

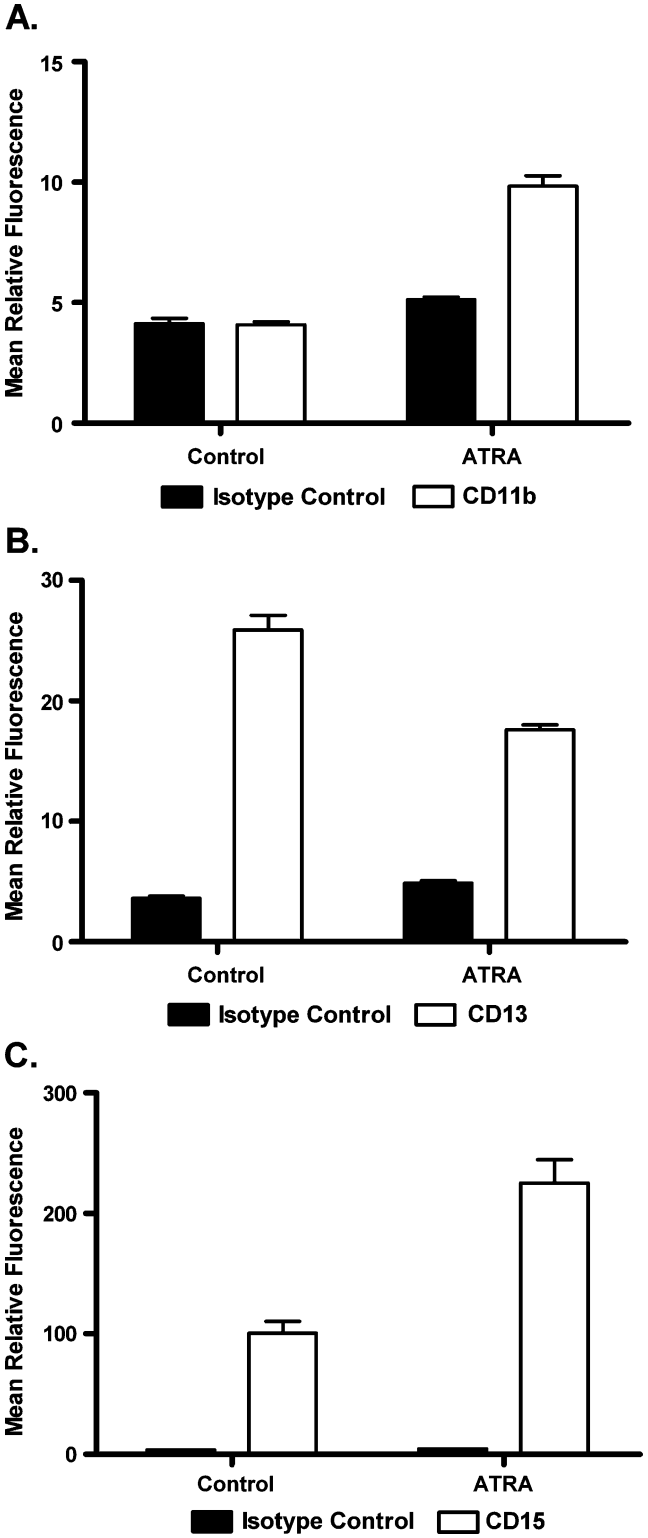

Figure 4. Measurement of cell surface antigen expression in ATRA-treated and vehicle-control HL-60 cells. Flow cytometry was used to measure changes in the expression of CD11b, CD13, and CD15 using cells that were treated for 5 d with 1 μM ATRA or vehicle-control. *A*. CD11b and isotype control IgG2b, *B*. CD13 and isotype control IgG1, *C*. CD15 and isotype control IgM. The data are shown as the mean±SE.

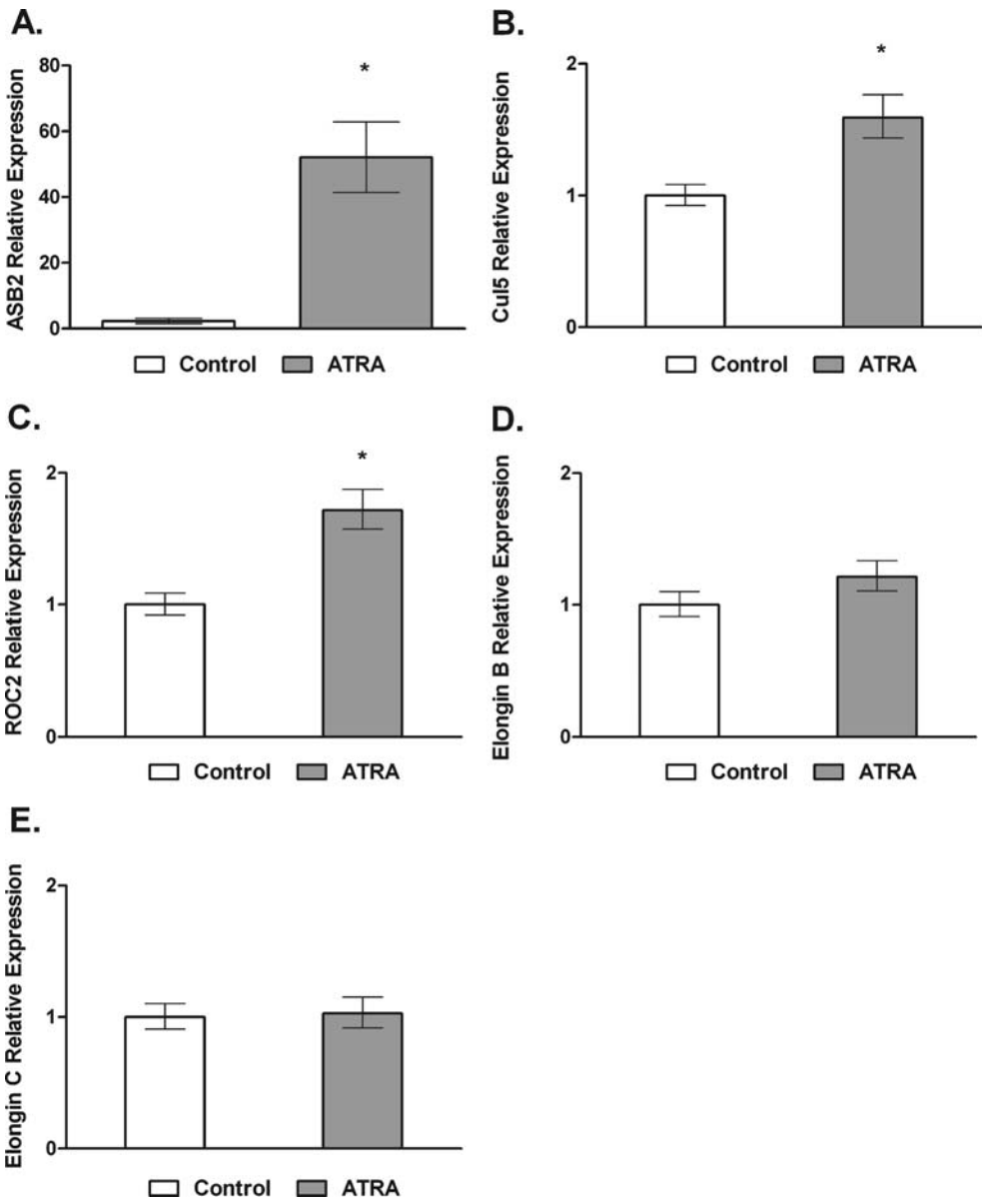

Figure 5. Real-time RT-PCR analysis of ASB-2, Cul5, ROC2, elongin B, and elongin C mRNA expression during ATRA-mediated granulocytic differentiation of HL-60 cells. Cells were treated for 5 d with 1 μM ATRA or vehicle-control and real-time RT-PCR was performed using Taqman® Gene Expression Assays. *A*. Relative expression of ASB-2 mRNA determined using the relative standard curve method. The data are shown as the normalized mean quantity for the ATRA-treated cells relative to the normalized mean quantity of the vehicle-control cells±SD; *B*. relative expression of Cul5 mRNA determined using the $\Delta\Delta C_T$ method. The data are shown as the fold difference calculated from the equation $2^{-\Delta\Delta CT}$±SD; *C*. relative expression of ROC2 mRNA determined using the $\Delta\Delta C_T$ method. The data are shown as the fold difference calculated from the equation $2^{-\Delta\Delta CT}$±SD; *D*. relative expression of elongin B mRNA determined using the $\Delta\Delta C_T$ method. The data are shown as the fold difference calculated from the equation $2^{-\Delta\Delta CT}$±SD; *E*. relative expression of elongin C mRNA determined using the $\Delta\Delta C_T$ method. The data are shown as the fold difference calculated from the equation $2^{-\Delta\Delta CT}$±SD; n=9 per group; * statistically significant at $P \leq 0.05$ using two-tailed Student's t test.

predicted molecular weight by Western blot analysis (*data not shown*).

Discussion

The expression of ASB-2 mRNA has previously been shown to be induced with ATRA treatment of APL cells (Kohroki et al. 2001; Guibal et al. 2002). The ASB-2 protein is known to interact with Cul5, elongin B/C, and ROC2 to form an ECS E3 ubiquitin ligase (Heuze et al. 2005; Kohroki et al. 2005). The purpose of this study was to determine if there is increased expression of Cul5 during ATRA-induced granulocytic differentiation of HL-60 cells. As a model of granulocytic differentiation, we treated HL-60 cells with ATRA (1 μM) for 5 d (Mayer et al. 1996). Differentiation was confirmed by observing the expected changes in nuclear morphology (Olins and Olins 2004), an increase in PMA-stimulated superoxide anion production (Mayer et al. 1995), and the expected changes in the expression of the cell surface antigens CD11b, CD13, and CD15 (Brackman et al. 1995; Trayner et al. 1998). Quantitative real-time RT-PCR was used to analyze mRNA expression of the ECS E3 ubiquitin ligase components, ASB-2, Cul5, ROC2, and elongin B and C, and Western Blot analysis was used to examine protein expression of Cul5 in ATRA-differentiated HL-60 cells.

Within these ECS E3 ubiquitin ligase complexes, Cul5 serves an important role to bring in close proximity an E2 ubiquitin conjugating enzyme with the substrate recognition components of the complex, by acting as a scaffold

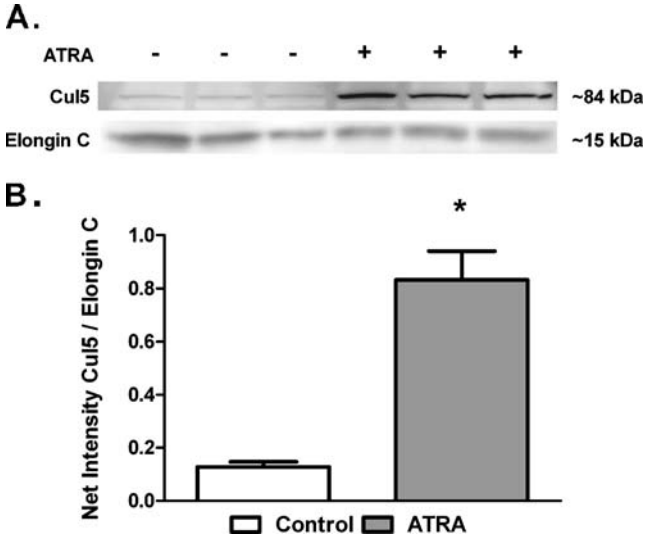

Figure 6. Western blot analysis of Cul5 expression during ATRA-mediated granulocytic differentiation of HL-60 cells. Cells were treated for 5 d with 1 μM ATRA or vehicle-control and 50 μg of protein per sample was subjected to SDS-PAGE. *A*. Blots were probed using a rabbit anti-Cul5 polyclonal antibody or a rabbit anti-elongin C polyclonal antibody; representative results from three vehicle-control and three ATRA-treated samples are shown. *B*. Cul5 protein expression significantly increased 6.5-fold in the ATRA-treated cells versus the vehicle controls when using elongin C. to normalize for loading differences. The data are shown as the mean net intensity of Cul5 divided by the mean net intensity of elongin C±SE; $n=6$ per group; * statistically significant at $P\leq 0.05$ using two-tailed Student's t test.

(Hershko and Ciechanover 1998; Deshaies 1999; Kile et al. 2002; Willems et al. 2004). Cul5 is a member of the cullin gene family based on the presence of a Carboxyl-terminal Cul domain; other members of this family include Cul1, Cul2, Cul3, Cul4A, Cul4B, Cul7, KIAA0708, Parc, and APC2 (Burnatowska-Hledin et al. 1995; Kipreos et al. 1996; Mathias et al. 1996; Yu et al. 1998; Dias et al. 2002; Nikolaev et al. 2003). In the present study, Cul5 mRNA and protein expression increased 1.6-fold and 6.5-fold, respectively, in ATRA-treated HL-60 cells versus vehicle-control cells, and this increase was associated with granulocytic differentiation. An increase in Cul5 expression with granulocytic differentiation is not totally unexpected, as overexpression of Cul5 inhibits proliferation in CHO, COS-1, and T47D cell lines (Van Dort et al. 2003; Burnatowska-Hledin et al. 2004), and Cul5 plays a role in cell fate determination in *Drosophila* (Ayyub et al. 2005). Additional studies also support a putative tumor suppressor role for Cul5 in breast cancer, as the chromosomal location (11q22-23) for Cul5 is associated with loss of heterozygosity (Carter et al. 1994; Hampton et al. 1994; Gudmundsson et al. 1995; Negrini et al. 1995; Tomlinson et al. 1995; Winqvist et al. 1995; Byrd et al. 1997; Driouch et al. 1998) and there is decreased expression of Cul5 in breast tumor tissues versus matched normal tissues (Fay et al. 2003). A 1.6-fold change in Cul5 mRNA expression, and a 6.5-fold change in Cul5 protein expression, may have an important role in cellular processes such as differentiation since a previous study indicated that a less than twofold increase in the expression of another cullin family member, Cul4A, promotes proliferation and attenuates granulocytic differentiation of the PLB-985 human myeloid leukemia cell line (Li et al. 2003). Other studies have shown similar fold changes in Cul5 expression in some rat tissues following hemorrhagic shock, traumatic brain injury, and osmotic stress (Ceremuga et al. 2003a, b; Yao et al. 2006). It is also of interest that a recent study showed a decrease in Cul5 mRNA expression in B-cell chronic lymphocytic leukemia (Kalla et al. 2007).

Another important component of Cul5 containing E3 ubiquitin ligases is a ring finger protein, such as ROC2/sensitive to apoptosis gene (SAG)/Rbx2 (Ring Box)/Hrt2 (Ohta and Xiong 2001; Kohroki et al. 2005). The ring finger protein ROC2/SAG/Rbx2/Hrt2 belongs to an evolutionarily conserved protein family that also includes ROC1/Rbx1/Hrt1 (Duan et al. 1999; Kamura et al. 1999; Ohta et al. 1999a; Seol et al. 1999). In E3 ubiquitin ligase complexes, the ROC protein binds to a cullin protein to facilitate the interaction between the E2 ubiquitin conjugating enzyme and the E3 ubiquitin ligase (Hershko and Ciechanover 1998; Deshaies 1999; Willems et al. 2004). The present study shows that ROC2 mRNA expression increased 1.7-fold in ATRA-treated HL-60 cells versus vehicle-control cells, and this increase was associated with granulocytic differentiation. The finding of a similar fold increase with ATRA-treatment for ROC2 and Cul5 mRNA expression may be important since association of ROC proteins with cullins has been shown to protect ROC proteins from degradation (Ohta et al. 1999b). ROC2 has been reported to be involved in cellular processes such as proliferation and apoptosis and to play a role as a stress responsive gene (Sun et al. 2001). ROC2 was identified as a gene induced by the redox-sensitive compound, 1,10-phenanthroline, in mouse tumor cell lines using the method of differential display (Duan et al. 1999; Sun et al. 2001). The induction of ROC2 expression by redox-sensitive agents has been shown to protect cultured cells in vitro from apoptosis (Duan et al. 1999) and to protect mouse neurons from apoptosis in an ischemia/reperfusion in vivo mouse model (Yang et al. 2001). As with Cul5, ROC2 has also been implicated in cancer, since a recent study demonstrated that ROC2 attenuates 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-mediated transformation by a mechanism that involves ubiquitination and degradation of c-Jun (Gu et al. 2007).

Elongin B and C are also key components of Cul5-containing ECS E3 ubiquitin ligases in which they act as adapter proteins that link the SOCS box protein to the

cullin-ROC-E2 portion of the complex (Kibel et al. 1995; Kamura et al. 1998; Iwai et al. 1999; Kamura et al. 2001). Elongin B and C serve a dual cellular role since they were originally shown to interact with elongin A to form transcription factor SIII, which augments transcription by promoting the elongation activity of RNA polymerase II (Bradsher et al. 1993a, b; Aso et al. 1995). We found no significant changes in mRNA expression for the adapters, elongin B and C, in the ATRA-treated HL-60 cells.

In the present study, ASB-2 served as a positive control since previous research showed that ASB-2 is a transcriptional target of ATRA during granulocytic differentiation of HL-60 cells (Kohroki et al. 2001; Guibal et al. 2002). As expected, ASB-2 mRNA expression was significantly increased 23-fold in the ATRA-treated cells versus the vehicle-control cells. ASB-2 belongs to a family of proteins that contain a carboxyl protein motif called the SOCS box (Hilton et al. 1998; Kile et al. 2000). In E3 ubiquitin ligase complexes, these SOCS box proteins act as the substrate recognition protein, and as such identify the ubiquitination target proteins (Deshaies 1999; Kile et al. 2002; Willems et al. 2004). A recent study demonstrated that knockdown of ASB-2 delays ATRA-mediated differentiation of PLB-985 myeloblastic leukemia cells, and that the actin binding proteins, filamin A and B, are targeted for proteosome-mediated degradation by the substrate recognition protein ASB-2 (Heuze et al. 2008).

In conclusion, we found that Cul5 mRNA and protein expression are significantly increased with ATRA-mediated granulocytic differentiation of HL-60 cells. These results build upon previous findings demonstrating that ASB-2 expression is induced during ATRA-mediated granulocytic differentiation of HL-60 cells (Kohroki et al. 2001; Guibal et al. 2002). The mechanism by which ATRA increases ASB-2 expression is attributed to a RARE-like sequence in the promoter region of the *Asb-2* gene (Kohroki et al. 2001). In this study, it is not clear if the increase in Cul5 and ROC2 mRNA expression is mediated via RAREs in the promoter regions of these genes or results from an indirect effect. The increase in Cul5 protein expression may reflect both increased transcription and increased protein stability/decreased degradation and warrants further investigation. In addition to components of the Cul5-containing ECS E3 ubiquitin ligase complex being induced by ATRA in APL cells, previous research indicates that the transcription of an E1-like ubiquitin-activating enzyme, UBE1L, is increased by ATRA (Tamayo et al. 1999; Kitareewan et al. 2002). The fact that multiple components of the Cul5 containing ECS E3 ubiquitin ligase complex are significantly increased with ATRA-mediated granulocytic differentiation of HL-60 cells suggests an important role for this E3 ubiquitin ligase in granulocytic differentiation and differentiation therapy.

Acknowledgments We thank the Robert H. Lurie Comprehensive Cancer Center Flow Cytometry Core Facility at Northwestern University and Dr. Alice Givan of the Englert Cell Analysis Laboratory at Dartmouth Medical School for assistance with the collection and analysis of the flow cytometry data. We also thank Dr. Nalini Chandar of the Biochemistry Department at Midwestern University for reviewing this manuscript prior to submission. This research was supported in part by the Biomedical Sciences Department and the Office of Research and Sponsored Programs at Midwestern University and by grant number R15 CA 122003-01 from the National Cancer Institute of the NIH.

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