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Retroviral Mediated Expression of the Human Myeloid Nuclear Antigen in a Null Cell Line Upregulates *Dlk1* Expression

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Abstract The human myeloid nuclear differentiation antigen (MNDA) is a hematopoietic cell specific nuclear protein. MNDA and other related gene products interact with and alter the activity of a large number of proteins involved in regulating specific gene transcription. *MNDA* and related genes exhibit expression characteristics, which suggest functions unique to specific lineages of cells, in addition to mediating the effects of interferons. Cells of the human K562 myeloid line do not express *MNDA* and are relatively immature compared to lines that express *MNDA* (HL-60, U937, and THP1). The hypothesis that MNDA influences the expression of specific genes was tested by creating *MNDA* expressing K562 cells using stable retroviral mediated gene transfer followed by evaluation of transcription profiles. Two macroarrays containing a total of 2,350 cDNAs of known genes showed a specific up-regulation of *Dlk1* expression in *MNDA* expressing K562 cell clones. Real time quantitative RT-PCR analysis confirmed an average of over 3- and 7-fold upregulation of *Dlk1* in two clones of *MNDA* expressing K562 cells. The effects on *Dlk1* were also confirmed by Northern blotting. *Dlk1* is essential for normal hematopoiesis and abnormal expression is a proposed marker of myelodysplastic syndrome. Additional screening of transcription profiles after induced erythroid and megakaryoblastic differentiation showed no additional gene transcripts altered by the presence of MNDA. These results indicate that MNDA alters expression of a gene essential for normal hematopoiesis. J. Cell. Biochem. 86: 56–66, 2002.

Key words: MNDA; K562; transcription profiles; *Dlk1* expression; hematopoiesis; myelodysplastic syndrome

The human myeloid nuclear differentiation antigen, *MNDA*, is a member of a family of interferon-regulated genes. The human genes (*MNDA*, *IFI16*, and *AIM2*) and the mouse genes (*202a*, *202b*, *203*, *204*, and *D3*) share one or two copies of a partially conserved 200 amino acid coding sequence (*IFI 200* gene family) [Johnstone and Trapani, 1999]. Whereas interferon induction or upregulation is a common characteristic of all these genes, they also appear to exhibit high levels of constitutive expression in specific cell types, which suggests functions associated with lineage specific differentiation. The most restricted pattern of expression is exhibited by *MNDA* for which detection is limited to human hematopoietic cells, including maturing cells of the myelomonocytic lineage and a subset of mature B cells showing low levels of expression [Miranda et al., 1999]. Alterations in the levels of *MNDA* expression in monocytic cells suggest a role in inflammation and foam cell function [Miranda et al., 1999; Shiffman et al., 2000]. Unlike other genes in the family, *MNDA* upregulation by interferon is limited to only certain of the cells that exhibit a

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constitutive level of expression [Briggs et al., 1994]. Expression of other genes in the family is not limited to hematopoietic cells, despite which specific roles in hematopoietic cell differentiation have been proposed [Tannenbaum et al., 1993; Dawson et al., 1998; Gariglio et al., 1998, 1999; Weiler et al., 1999]. In addition, the mouse proteins 202a and 204 are thought to be important in skeletal muscle differentiation [Datta et al., 1998; Liu et al., 2000]. Other observations suggest roles for genes in this family in viral replication [Hertel et al., 1999], tumor suppression [DeYoung et al., 1997; Wen et al., 2000, 2001], and autoimmunity [Seelig et al., 1994; Rozzo et al., 2001].

Proteins encoded by genes in the *IFI 200* family generally localize in the nucleus with the exception of AIM2, which is cytoplasmic [Choubey et al., 2000]. In addition, p202a is both cytoplasmic and nuclear, but after interferon treatment localizes predominantly in the nucleus [Choubey and Lengyel, 1993]. The nuclear localization of the remaining members of the family, combined with the characterization of biochemical properties and assessment of their effects on cell physiology, has led to the hypothesis that members of this gene family influence transcription of specific genes. The *IFI16* gene fused to the GAL4 DNA binding domain repressed a reporter construct with a thymidine kinase promoter and GAL4 DNA elements [Johnstone et al., 1998]. However, the mechanism whereby members of the family might regulate gene transcription remains unclear, as none of these proteins bind DNA in a sequence-specific fashion and only two (IFI16 and p202a) have been reported to bind DNA directly in a nonsequence-specific fashion [Choubey and Lengyel, 1992; Dawson and Trapani, 1995; Choubey and Gutterman, 1996; Luu and Fores, 1997; Xie et al., 1997].

The proposal that MNDA and other products of this gene family bind to and modulate the action of other proteins involved in regulating transcription is supported by a number of observations. MNDA binds nucleolin, nucleophosmin and YY1 [Xie et al., 1995, 1997, 1998]. YY1 is a multifunctional transcription factor that also interacts with both nucleophosmin and nucleolin. These associations alter YY1 transcriptional regulatory activity [Inouye and Seto, 1994; Lee and Lee, 1994; Yang et al., 1994]. MNDA binding stimulates YY1 binding to its target DNA element. Certain of the other members of the *IFI 200* gene family interact or interfere with the function of transcription regulatory proteins including c-Fos, c-Jun, AP2, E2F1, E2F4, Myo D, myogenin, P53, P53BP1, Rb, NF-kB p50 and p65, UBF1 and c-Myc [Liu et al., 1999; Johnstone et al., 2000; and reviewed in Johnstone and Trapani, 1999]. The effects of p202a or p204 on c-Myc, UBF1, NF-kB, c-Fos, c-Jun, p53BP1, Rb, E2F1, and MyoD activity were also associated with downregulation of appropriate endogenous genes [Choubey et al., 1996; Datta et al., 1996, 1998; Min et al., 1996; Gutterman and Choubey, 1999; Liu et al., 1999; Hertel et al., 2000; Wang et al., 2000]. The interaction of proteins in the IFI 200 family with transcription regulator proteins has generally been mapped to the conserved 200 amino acid region, which appears in one or two copies in all members. The majority of the work characterizing functionally significant interactions with transcription regulatory proteins has centered on the mouse p202a. In addition, in some of these studies, the related p204 did not interact with the same proteins. Therefore, it is unclear if any interaction or function is common to all members of this family of genes.

Forcing the expression of *MNDA* in a *MNDA* non-expressing cell might alter the levels of specific transcripts of potential targets. We tested this hypothesis by introducing *MNDA* into the nonexpressing K562 cell line using a retroviral vector and evaluating transcription profiles using DNA macroarrays. Levels of constitutively expressed genes, and those associated with erythroid and megakaryoblastic differentiation were evaluated. Our results indicate that MNDA upregulates *Dlk1*, a gene essential for normal hematopoietic cell differentiation.

MATERIALS AND METHODS

Cells, Treatments, Isolations, and Assays

K562 (ATCC CLL-243) was cultured in RPMI 1640 medium supplemented with 15% fetal bovine serum and antibiotics. Bosc 23 [Pear et al., 1993] and PA317 (ATCC CRL-9078) cells were grown in DMEM with 10% fetal bovine serum and antibiotics. All cell lines were grown in a humidified, 37°C incubator in 5% CO_2. PMA (TPA, 12-*O*-tetradecanoylphorbol 13-acetate, Sigma-Aldrich, St. Louis, MO) was solubilized in ethanol and added to cell cultures in rapid growth phase for the times and concentrations

specified. AraC (Cytosine arabinoside, Sigma-Aldrich) was dissolved in H_2O and added to growing cells at a final concentration of 100 nM for 24 h. Total RNAs were extracted from cells using the Trizol™ reagent (Invitrogen Living Science, Carlsbad, CA). After precipitation with isopropanol, samples were washed with 75% ETOH and then rehydrated in a small volume of RNase free H2O (50 μl/10 million cells) prior to additional purification using a resin based column procedure (Nucleospin RNA II, Clontech Laboratories, Inc., Palo Alto, CA). RNA was quantified by OD260 absorption readings and then analyzed by formaldehyde agarose gel electrophoresis. Intact 18S and 28S ribosomal RNA bands were observed using these procedures. Details of the immunoblot and immunocytochemical staining procedures have been provided previously [Briggs et al., 1994; Miranda et al., 1999].

Northern Blots

Full length *Dlk1* cDNA and 1.1kb *G3PDH* fragments were amplified by RT PCR from K562 cell RNA with Advantage II DNA polymerase mixture (Clontech) and subcloned using TOPO T/A cloning kit (Invitrogen). Clones containing the correct sized inserts were selected and sequenced. Plasmid DNA containing *Dlk1* and *G3PDH* inserts was digested with restriction enzymes. The inserts were isolated by agarose gel separation. The insert DNA was extracted from the gel and purified using Nucleospin column (Clontech). *Dlk1* (250 ng) or *G3PDH* (50 ng) probes were labeled with fluorescein for nonradioactive ECL detection following the manufacturer's procedure (Amersham Biosciences Corp., Piscataway, NJ). Total RNA was separated on 1% denaturing formamide agarose gel and transferred in ten times SSC onto charged nylon membranes (Hybond N^+, Amersham Biosciences). Blots were prehybridized with QuickHyb hybridization solution (Stratagene, La Jolla, CA) and hybridized overnight at 45°C. Blots were washed at 55°C twice with 1 × SSC/0.5% SDS and once with 0.5 × SSC/0.5% SDS. Hybridization signals were detected using ECL reagents (Amersham Biosciences) and analysis performed on a Macintosh computer using the public domain NIH Image program (developed at the U.S. National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/nih-image/).

Retroviral Transfer of *MNDA* cDNA Into K562 Cells

Sequence encoding the entire *MNDA* open reading frame was inserted into the MMLV derived defective vector LXSN [Miller and Rosman, 1989] and sequenced to confirm correctness of ligated junctions. pLXSN-MNDA and pLXSN were transfected into the ecotropic Bosc 23 packaging line and the viral supernatants from the Bosc 23 cultures were used to transduce the amphotropic PA317 packaging cell line. G418 resistant PA317-MNDA cloned cells were tested for ability to express MNDA by Western blot. Both the LXSN-MNDA and the empty vector (LXSN) PA317 cloned cell lines exhibiting the highest titer were then used to transduce K562 cells by co-cultivation in three different Transwell™ plates (Corning Inc., Harrodsburg, KY). Neomycin resistant K562 populations infected with the LXSN-MNDA retrovirus were cloned by two rounds of limiting dilution, expanded and selected for MNDA protein expression by Western blot. Two clones, designated K562 LXM-A2 and K562 LXM-D2, were subcloned from different original co-cultivation plates. MNDA protein expression levels were similar to those observed in constitutively expressing cell lines (U937, HL-60, and THP-1). Control vector transduced cells, K562 LXC, are a mixed population of G418 resistant cells. The same procedures were used to transduce the KG-1 (ATCC, CCL-246) human myeloblastic leukemia cell line, but all neomycin resistant clones that were isolated failed to express MNDA.

cDNA Array Analysis

Atlas Human 1.2 and Atlas Cancer 1.2 (Clontech) nylon based membrane cDNA macroarrays were used to evaluate transcription profiles. Four identical membranes of each array were developed and analyzed simultaneously to detect differential message expression using probes prepared from the K562 parental and retroviral cloned cell lines. Each membrane is spotted with a total of 1,176 genes and 9 housekeeping genes (Clontech). A list of all of the genes represented on both of these arrays is available on the Clontech web site (www.clontech.com/atlas). ^{32}P-labeled probes were prepared by first strand cDNA synthesis using primers complementary to the immobilized cDNAs according to the procedures

recommended by the manufacturer. Probes were purified using Nucleospin Extraction columns (Clontech) and then alkali denatured. The entire labeled product was hybridized to a single membrane for 18 h at 68°C in Express Hyb hybridization solution. Membranes were washed, sealed in plastic and exposed to phospho-imaging screens for 1–3 days. Signals were quantified using the Cyclone Imaging system and the OptiQuant analysis software (Packard Instrument Company, Meriden, CT). Background corrections were made within images. Between image corrections were based on the sum of signal output for all 9 housekeeping genes. A program was designed in Microsoft Excel whereby data for all four arrays could be compared both numerically and graphically.

Real Time Quantitative RT-PCR

Real time quantitative RT-PCR was carried out using the ABI Prism 7700 Sequence Detection System (ABI, Applied Biosystems, Foster City, CA) with the Taqman One-Step RT-PCR protocol and reagents (ABI). The Ribosomal RNA Control (18S) endogenous system (Vic-reporter/Tamra labeled probe, ABI) was used for normalization. Gene regions selected for designing amplimers were limited to the specific cDNA sequence used on the Atlas array. Amplimer size and sequences for primers and probes were selected in accordance with guidelines suggested in ABI protocols and aided by the Primer Express Software program (ABI). Custom primers were synthesized by the DNA Core facility at Vanderbilt University Medical Center and the Fam (reporter)/Tamra fluorescent labeled internal probes were prepared by the ABI Oligo Factory. Prior to real time assay, each set of primers, along with the 18S primer set, were tested for ability to produce the expected size end product by standard RT-PCR. These reactions included a no RT control to test for possible contaminating genomic DNA. None of the RNAs prepared by the two-step extraction purification method used in this study showed evidence of significant DNA contamination. The products were visualized by electrophoresis on an 8% vertical acrylamide/TAE gel. Real time assays were performed in ABI Prism 96 well plates with optical adhesive covers (ABI), 25 μl per well and each sample in triplicate. Primer, probe, and RNA concentrations were optimized for each selected amplimer. Results were analyzed using the SDS Version 1.7 software and the ΔΔCt method as described in ABI protocols (Applied Biosystems, 1997). In the case of *Dlk1*, the Real Time quantitative RT-PCR was performed three times with different RNA preparations.

RESULTS

MNDA Expression in Retrovirally Transduced K562 Clones

MNDA expression in the stable retrovirally transduced K562 LXM-D2 clone was examined by immunoblot assay, and the level was comparable to that in the U937 cell line (Fig. 1, compare lanes 1 and 3). The level of MNDA in the K562 LXM-A2 clone was similar to the D2 clone (data not shown). Phorbol ester treatment at two doses did not alter MNDA expression in the K562 LXM-D2 clone (Fig. 1, compare lanes 5, 7, 11, and 13 to lanes 3 and 9). The results indicate that in the retrovirally transduced cells, *MNDA*

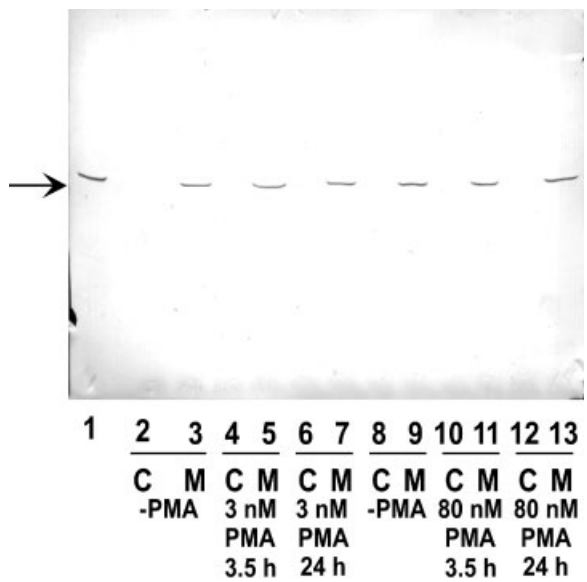

Fig. 1. Immunoblot analysis of K562 LXM-D2 transduced clone expessing a full length *MNDA* cDNA. This clone expressed similar levels of MNDA protein to the U937 cell line, which expresses *MNDA* constitutively. Cell lysates were prepared from phorbol ester treated or untreated cells and 5×10^4 lysed cells were loaded in each lane. Lane 1 corresponds to total protein from the U937 cell line (arrow). Even-numbered lanes (C) correspond to total cellular proteins from the *MNDA* nonexpressing K562 cells transduced with the empty retroviral vector. Odd numbered lanes (M) contain total cellular proteins from the *MNDA* expressing K562 LXM-D2 retroviral transduced clone. Cells untreated are in lanes 1, 2, 3, 8, and 9. Cells treated with phorbol ester, for the times and concentrations specified are in lanes 4, 5, 6, 7, 10, 11, 12, and 13.

expression originates from retroviral regulatory sequences, which are unaffected by phorbol ester. The same treatment completely repressed *MNDA* mRNA in cells that express a constitutive level of *MNDA* (Briggs et al., 1994). Immunocytochemical staining of cells in the K562 LXM-D2 clone showed that all cells express MNDA, although the level appears to vary between cells (Fig. 2). The isolation of retrovirally transduced cell clones that express relatively high levels of *MNDA* indicated that its expression did not significantly suppress growth of K562 cells. No differences in cell counts were observed through repeated growth cycles of K562 parental cells and the *MNDA* expressing clones (data not shown). Two mixed cell cultures were established by combining K562 parental untransduced cells with equal numbers of *MNDA* expressing K562 LXM-D2 clone. The percent of MNDA positive cells in the mixed cultures at the end of two years was 60 and 28. Thus, no consistent effect on cell proliferation was observed. Attempts to transduce KG-1 human myeloblastic cells with the *MNDA* expressing retroviral construct were unsuccessful. None of the 90 neomycin resistant clones of KG-1 cells examined expressed *MNDA*.

Transcription Profiles in MNDA Expressing or Nonexpressing K562 Cells

The transcription profiles of constitutively expressed genes from K562 parental., K562 LXC (control) and K562 LXM-A2 and K562 LXM-D2 (MNDA-expressing) cells, overall, were very alike, showing similar level and pattern of positive and negative signals. Evidence that the approach could detect transcription differences was obtained from the analysis of the Atlas human 1.2 macroarray, which contains an *MNDA* cDNA. In this case, signal was observed only in the two *MNDA* expressing clones of K562 (Fig. 3). The difference in *MNDA* signals between the four lines, despite originating from retrovirally manipulated transcripts, confirmed that gene expression differences between the two *MNDA* expressing K562 clones and the two control K562 cell lines could be detected. Analysis of the array signals on both the Atlas human 1.2 and human cancer 1.2 showed noticeable increased *Dlk1* transcript signal in *MNDA* expressing cells (Fig. 4). Transcription profiles were also evaluated after phorbol ester treatment of the four cell lines to induce megakaryoblastic differentiation or after AraC treatment to induce erythroid differentiation. The *Dlk1* signals in the *MNDA* expressing cells averaged over four-fold higher in the presence of differentiation inducers (Fig. 5).

A number of less obvious differences was detected between *MNDA* expressing K562 cells and the nonexpressing control cells. Four of the

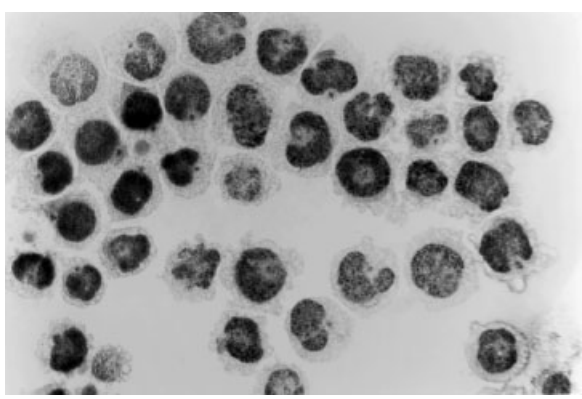

Fig. 2. Immunoperoxidase detection of MNDA in nuclei of D2 clone of retrovirally transduced K562 cells (original magnification × 630).

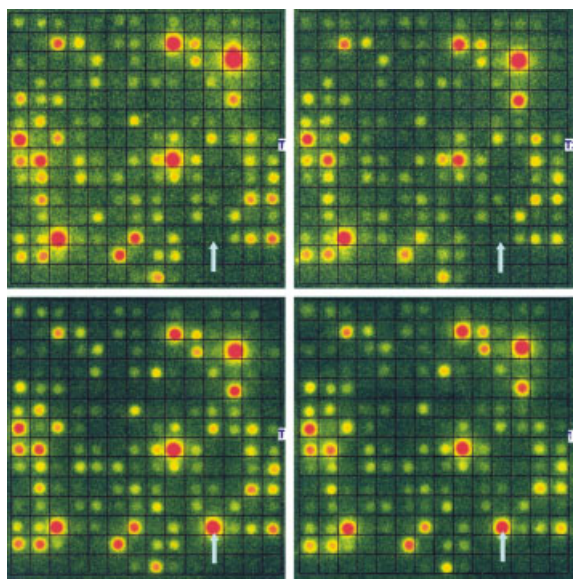

Fig. 3. Transcription profile using Atlas human broad array 1.2 I. One of six quadrants (196 cDNAs) shown from 1,176 cDNA array of known genes probed with labeled cDNAs from K562 parental cells (upper left), K562 transduced with empty retroviral vector (upper right), or *MNDA* expressing clones of transduced K562 cells (lower panels). The *MNDA* signal (arrow in each panel) is significantly higher in both *MNDA* expressing K562 cloned lines (lower panels). Note the very similar levels of signals for the remaining 195 genes between the four panels. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

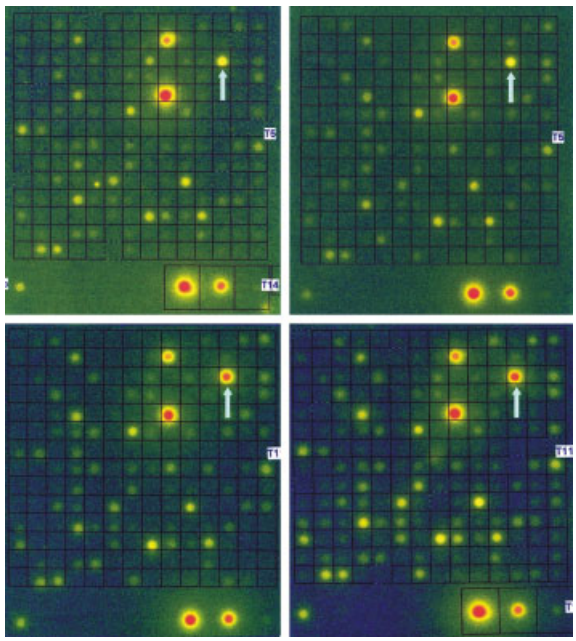

Fig. 4. Transcription profile using Atlas cancer array 1.2. One of six quadrants (196 cDNAs) shown from 1,176 cDNA array of known genes probed with labeled cDNAs from K562 parental cells (upper left), K562 transduced with empty retroviral vector (upper right), or *MNDA* expressing clones of transduced K562 cells (lower panels). The *Dlk1* signal (arrow in each panel) is significantly higher in both *MNDA* expressing K562 cloned lines (lower panels). Note the very similar levels of signals for the remaining 195 genes between the four panels. The results provide an indication of the specificity of the effect of MNDA on *Dlk1*. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

most highly altered transcripts (transcripts showing a minimum two-fold difference between K562 LXM-A2 and K562 LXM-D2 compared to K562 parental and K562 LXC empty vector control) were selected for further analysis (Table I).

Confirmation of Differences in Selected Transcript Expression

Real time quantitative RT PCR analysis of the *Dlk1* transcript in the K562 cells showed an average of over 3- or 7-fold increased expression in clones of *MNDA* expressing K562 cells relative to parental cells or empty vector control K562 cells (Table II and III). The real time analysis of the other selected genes (Table I) did not show a consistent difference in expression levels between the MNDA positive and negative K562 cells. A Northern blot analysis of *Dlk1* transcript levels (Fig. 6) showed an average of over 4-fold increased expression in clones of MNDA expressing K562 cells relative to parental K562 cells.

DISCUSSION

K562 cell clones transduced with a retroviral vector and stably expressing *MNDA* exhibited a dramatically altered steady state level of *Dlk1* transcript. *Dlk1* is a member of the EGF-like homeotic protein family, which includes Notch

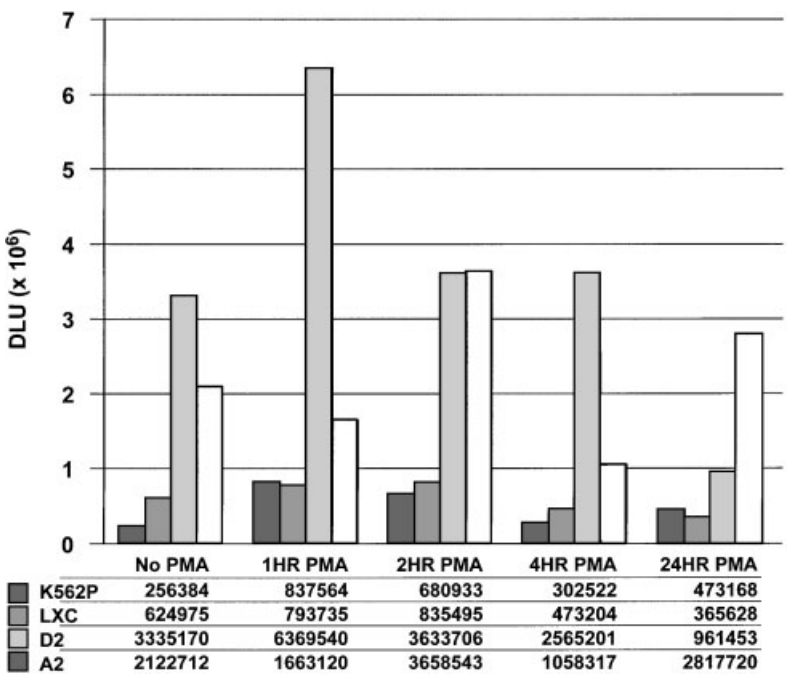

Fig. 5. Levels of *Dlk1* transcripts derived from quantitation of arrays after correction for background and housekeeping gene signals. Level of *Dlk1* in the *MNDA* expressing clones (K562 LXM-D2 and K562 LXM-A2) were consistently elevated over 24 h of phorbol ester treatment (80 nM).

TABLE I. Suspected Altered Transcripts

Gene	Fold change[a]	Expression characteristics
ApoE	6 ×	Increase in MNDA expressing K562 cells over nonexpressing cells after 24 h of AraC treatment
NPM (B23)	2 ×	Increase in constitutive levels in MNDA expressing K562 cells over nonexpressing cells
Ear 2	2 ×	Increase in constitutive levels in MNDA expressing K562 cells over nonexpressing cells
Elf 1	5 ×	Increase in MNDA expressing K562 cells over nonexpressing cells after AraC treatment

[a]Derived from quantitation of array signals.

receptors and ligands. *Dlk1* has been implicated in adipogenesis, hematopoiesis, and neuroendocrine differentiation as well as tumorigenesis of specific cell types [Laborda, 2000]. Whereas the specific role of *Dlk1* in these processes remains unresolved, varying levels of *Dlk1* expression appear to play a role in the response of differentiating cells to extracellular stimulation [Laborda, 2000; Ruiz-Hidalgo et al., 2002]. Specifically, in neuroendocrine cell differentiation, changes in *Dlk1* levels appear to mark the decision to mature along different lineages. *Dlk1* was also proposed to influence differentiation signaling in hematopoiesis [Bauer et al., 1998]. *Dlk1* is produced by stromal cells and is essential for these cells to support hematopoiesis [Laborda, 2000]. The effects of *Dlk1* on the proliferation of hematopoietic progenitors has recently been described [Ohno et al., 2001]. While the initial emphasis on blood cell differentiation was focused on *Dlk1* expression in stromal cells, recent results show that a normal hematopoietic stem cell population expressed *Dlk1* and that it was selectively upregulated in the same cell population in myelodysplastic syndrome (MDS) [Miyazato et al., 2001]. The elevation of *Dlk1* was proposed as a marker of MDS and a contributor to ineffectual hematopoeisis, which is a hallmark of this disease. Our results showed that the K562 cell line which represents a multipotential progenitor cell also expressed *Dlk1* and that it was dramatically upregulated when *MNDA* expression was introduced into this cell. The biological consequences of the effect of MNDA on *Dlk1* in the K562 cell may reside in the cells, ability to respond to natural inducers to differentiate along specific blood cell lineages.

Our results showed that transcription profiles associated with chemical induction of differentiation were not altered by the presence of MNDA which might indicate MNDA does not play a role in regulating lineage-specific transcription, at least not in erythroid or megakaryoblastic development. An analysis of

TABLE II. Real Time Quantitative RT-PCR Analysis of *Dlk1* Transcripts in K562 Cells

	K562 Parental	K562 LXC	K562 LXM (D2)	K562 LXM (A2)
Experiment 1				
	27.01[a]	28.63	23.67	24.82
	26.89	28.37	24.22	24.78
	26.99	28.44	24.20	24.68
Mean	26.96	28.48	24.03	24.76
Std. err.	0.04	0.08	0.18	0.04
Experiment 2				
	25.04	26.51	22.43	23.78
	25.14	26.67	22.42	23.89
	25.22	26.69	22.47	23.78
Mean	25.13	26.62	22.44	23.82
Std. err.	0.09	0.10	0.02	0.10
Experiment 3				
	25.91	26.22	21.89	24.37
	25.79	26.23	23.06	24.13
	26.20	26.72	23.32	23.84
Mean	25.97	26.39	22.76	24.11
Std. err.	0.31	0.23	0.44	0.17

[a]Each RNA was evaluated in triplicate, and the ΔC_Ts provided represent sample C_Ts (PCR cycle where product signal exceeds threshold) corrected using 18S RNA CTs obtained from the same aliquot of RNA (Applied Biosystems, 1997). Three experiments were completed using three sets of RNAs isolated from MNDA expressing (K562 LXM-D2, K562 LXM-A2) and nonexpressing (K562 parental, K562 LXC) K562 cells.

TABLE III. The Average Fold Increase in *Dlk1* Transcript Levels for Both MNDA Expressing Clones Over the Non-Expressing K562 Parental Cells

	K562 LXM (D2)	K562 LXM (A2)
Fold change vs. K562P		
Experiment 1	7.62[a]	4.59
Experiment 2	6.46	2.49
Experiment 3	9.24	3.61
Mean	7.77	3.56

[a]$\Delta\Delta C_T$s were derived from the ΔC_Ts in Table II for each experiment and converted to fold difference between the MNDA expressing clones and the non-retroviral transduced K562 (parental) cells from each of three experiments (Applied Biosystems, 1997).

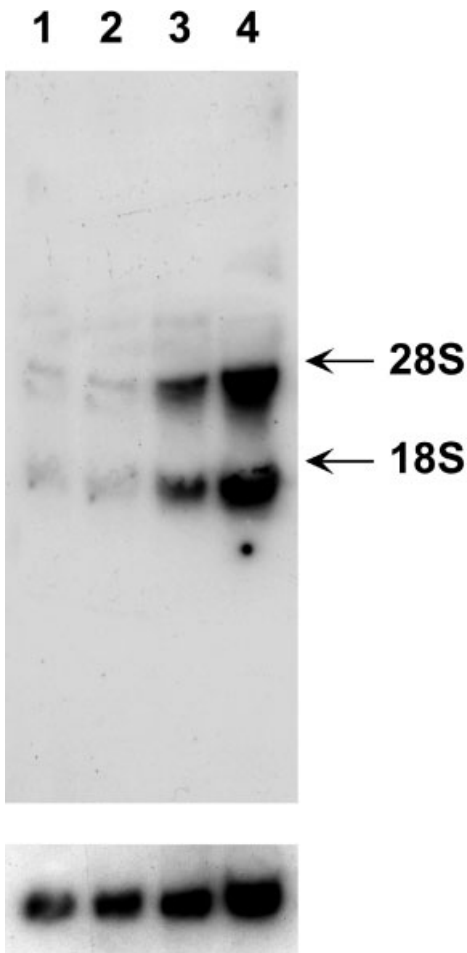

Fig. 6. Northern blot analysis of *Dlk1* (upper) and *G3PDH* (lower) mRNA levels. RNA from K562 parental (**lane 1**), K562 transduced with empty retroviral vector (**lane 2**), and *MNDA* expressing K562 cloned lines A2 (**lane 3**) and D2 (**lane 4**). The image analysis showed that *Dlk1* signals, after being corrected using *G3PDH* levels, in the *MNDA* expressing clones were more than 4-fold (A2) and 5-fold (D2) higher than in K562 parental cells.

transcription profiles based on array signals from cells treated with phorbol ester or AraC suggested that the induced differentiation was effective (data not shown). Phorbol ester treated K562 cells showed upregulated *MCL1*, *ERF1*, *ETR103*, *CD61*, and *TIMP1*, which is consistent with general cellular responses to phorbol ester and specifically, with megakaryoblastic differentiation of K562 cells [Alitalo et al., 1990; Shimizu et al., 1992; Kozopas et al., 1993; Bustin et al., 1994; Baker et al., 2001]. The effectiveness of AraC induction of erythroid differentiation was indicated by the downregulation of *GATA-2* [Ikonomi et al., 2000].

Dlk1 is expressed in a cell specific fashion and the downregulation associated with adipocyte differentiation occurred at the level of transcription [Laborda, 2000]. The downregulation of *Dlk1* in adipogenesis and in the developing adrenal cortex was used to justify a detailed analysis of the transcriptional regulation of the rat *Dlk1* [Takemori et al., 2001]. Sequence within a region containing two early growth response elements and a GC box near the transcription start site were found responsible for cell specific *Dlk1* promoter activity. The identity of the protein binding to that target sequence is unknown [Takemori et al., 2001]. The sequences upstream or downstream of the transcription start site in the rat, human, and bovine *Dlk1* gene are highly conserved suggesting that regulation of transcription is also conserved between species. MNDA and related gene products have been found to bind and influence the activity of a number of regulators of transcription. However, insufficient information is available to propose a mechanism whereby MNDA might upregulate *Dlk1* transcription. In addition, any of the proposed mechanisms of MNDA action would not be consistent with the experimental findings on gene products related to MNDA, which show that the interactions with transcription regulatory proteins result in repression of transcription of a large number of genes (discussed above). Opposite to this, our results showed that MNDA increased *Dlk1* expression and did not alter the level of a large number of genes.

Earlier work showed that MNDA binds the transcription factor YY1 and enhanced its ability to bind its target DNA element [Xie et al., 1998]. Whereas this interaction might account for the elevation of a YY1 targeted transcript, the possible role of YY1 in *Dlk1* trancription

is unknown. YY1 regulated ribosomal protein L32, c-fos, and alpha-globin, genes that are present on the arrays used in this study, did not show altered levels of transcripts in conjunction with the expression of *MNDA* in K562 cells. The significance of the interaction between MNDA and YY1 on regulation of transcription remains unknown. A large number of genes that are regulated by NF-κB are also present on the two arrays examined. The MNDA related mouse gene product p202a affects NFkB activity. However, NF-κB regulated genes, including *IL-1*, *IL-6*, *interferon gamma*, *p105*, *ICAM-1*, and *IL-2*, showed no change in transcript levels in MNDA expressing K562 clones (data not shown). Thus, it is unlikely that MNDA shares the ability of p202a to influence NF-κB activity. The absence of an effect of MNDA on YY1 or NF-κB regulated genes and the general lack of effect on nearly all of the other genes on the arrays indicates that MNDA has a very restricted role in regulating gene expression, even following phorbol ester treatment and induced differentiation. MNDA apparently does not influence the function of a large number of transcription factors, as is the case for p202a or IFI16, members of the IFI200 family. However, our results are based on screening less than 2,500 possible MNDA target regulated genes and it is likely that additional unknown target genes exist.

The expression of mouse genes related to *MNDA* has been reported to suppress cell proliferation [Johnstone and Trapani, 1999]. Converting the K562 cell line from nonexpressing to *MNDA* expressing did not result in reduced cell proliferation. However, repeated efforts to convert the KG-1 myeloblastic cell line from nonexpressing to *MNDA* expressing were unsuccessful. Whereas the KG-1 cells could be transduced by the retrovirus, none of the resistant clones isolated expressed *MNDA*. It is then possible that MNDA is a growth suppressor in specific cell types, including KG-1. Whereas both K562 and KG-1 are human leukemia cell lines that do not express *MNDA*, the K562 line is an earlier stage multipotential line, and KG-1 is a later stage myeloblastic line. A comprehensive analysis of *MNDA* expression in normal and neoplastic human tissues showed expression in both proliferating and nonproliferating hematopoietic cells, which is again inconsistent with attributing to MNDA a role in the control of cell proliferation [Miranda et al., 1999].

The known highly restricted expression of *MNDA* in hematopoietic cells is an indication that its function is associated with blood cell differentiation. *Dlk1* expression is essential for normal hematopoiesis, specifically for myeloid cell development. The recent discovery of abnormal *Dlk1* expression in MDS, a specific hematopathology defined by abnormal myeloid cell differentiation, further implicates *Dlk1* in hematopoiesis. Our observation that *MNDA* expression alters *Dlk1* expression in a hematopoietic cell line is suggestive of a novel regulatory mechanism with biological significance.

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REFERENCES

Alitalo R, Partanen J, Pertovaara L, Holtta E, Sistonen L, Andersson L, Alitalo K. 1990. Increased erythroid potentiating activity/tissue inhibitor of metalloproteinases and jun/fos transcription factor complex characterize tumor promoter-induced magakaryoblastic differentiation of K562 leukemia cells. Blood 75:1974–1982.

Applied Biosystems. 1997. Relative quantitation of gene expression. ABI PRISM 7700 Sequence Detection System. User Bulletin No. 2. Foster City, CA: PE Applied Biosystems.

Baker EJ, Ichiki AT, Day NE, Andrews RB, Bamberger EG, Lozzio CB. 2001. Simultaneous flow cytometric measurement of K-562 megakaryocytic differentiation and CD56 + large granular lymphocyte cytotoxicity. J Immunol Methods 253:37–44.

Bauer SR, Ruiz-Hidalgo MJ, Rudikoff EK, Goldstein J, Laborda J. 1998. Modulated expression of the epidermal growth factor-like homeotic protein dlk influences stromal-cell-pre-B-cell interactions, stromal cell adipogenesis, and pre-B-cell interleukin-7 requirements. Mol Cell Biol 18:5247–5255.

Briggs RC, Dworkin LL, Briggs JA, Dessypris EN, Stein GS, Stein JL, Lian J. 1994. Interferon alpha selectively affects expression of the human myeloid cell nuclear differentiation antigen in late stage cells in the monocytic but not the granulocytic lineage. J Cell Biochem 54:198–206.

Briggs RC, Kao WY, Dworkin LL, Briggs JA, Dessypris EN, Clark J. 1994. Regulation and specificity of MNDA expression in monocytes, macrophages, and leukemia/B-lymphoma cell lines. J Cell Biochem 56:559–567.

Bustin SA, Nie XF, Barnard RC, Kumar V, Pascall JC, Brown KD, Leigh IM, Williams NS, McKay IA. 1994. Cloning and characterization of ERF-1, a human member of the Tis11 family of early-response genes. DNA Cell Biol 13:449–459.

Choubey D, Gutterman JU. 1996. The interferon-inducible growth-inhibitory p202 protein: DNA binding properties and identification of a DNA binding domain. Biochem Biophys Res Commun 221:396–401.

Choubey D, Lengyel P. 1992. Interferon action: nucleolar and nucleoplasmic localization of the interferon-inducible 72 kDa protein that is encoded by the *Ifi204* gene from the gene 200 cluster. J Cell Biol 116:1333–1341.

Choubey D, Lengyel P. 1993. Interferon action: cytoplasmic and nuclear localization of the interferon-inducible 52kDa protein that is encoded by the *Ifi202* gene from the gene 200 cluster. J Interferon Res 13:43–52.

Choubey D, Li S-J, Datta B, Gutterman JU, Lengyel P. 1996. Inhibition of E2F-mediated transcription by p202. EMBO J 15:5668–5678.

Choubey D, Walter S, Geng Y, Xin H. 2000. Cytoplasmic localization of the interferon-inducible protein that is encoded by the AIM2 (absent in melanoma) gene from the 200-gene family. FEBS Lett 474:38–42.

Datta B, Li B, Choubey D, Nallur G, Lengyel P. 1996. p202, an interferon-inducible modulator of transcription, inhibits transcriptional activation by the p53 tumor suppressor protein, and a segment from the p53-binding protein 1 that binds to p202 overcomes this inhibition. J Biol Chem 271:27544–27555.

Datta B, Min W, Burma S, Lengyel P. 1998. Increase in p202 expression during skeletal muscle differentiation: inhibition of MyoD protein expression and activity by p202. Mol Cell Biol 18:1074–1083.

Dawson MJ, Trapani JA. 1995. The interferon-inducible nuclear antigen Ifi 16: localization to the nucleolus and identification of a DNA-binding domain. Biochem Biophys Res Commun 214:152–162.

Dawson MD, Elwood NJ, Johnstone RW, Trapani JA. 1998. Lineage-specific expression of the nucleoprotein IFI16 in monocyte but not polymorphonuclear leukocyte precursors in human bone marrow. J Leukoc Biol 64:546–554.

DeYoung KL, Ray ME, Johnstone RW, Su YA, Anzick SL, Meltzer PS, Trapani JA, Trent JM. 1997. Cloning of a novel member of the human interferon-inducible gene family associated with control of tumorigenicity in a model of human melanoma. Oncogene 15:453–457.

Gariglio M, De Andrea M, Lembo M, Ravotto M, Zappador C, Valente G, Landolfo S. 1998. The murine homolog of the HIN 200 family, Ifi 204, is constitutively expressed in myeloid cells and selectively induced in the monocyte/macrophage lineage. J Leukoc Biol 64:608–614.

Gribaudo G, Riera L, Hertel L, Landolfo S. 1999. In vitro and in vivo expression analysis of the interferon-inducible 203 gene. J Interferon Cytokine Res 19:129–136.

Gutterman JU, Choubey D. 1999. Retardation of cell proliferation after expression of p202 accompanies an increase in p21WAF1/CIP1. Cell Growth Differ 10:93–100.

Hertel L, Andrea MD, Azzimonti B, Rolle A, Gariglio M, Landolfo S. 1999. The interferon-inducible 204 gene, a member of the Ifi200 family, is not involved in the antiviral state induction by IFN-α, but is required by the mouse cytomegalovirus for its replication. Virology 262: 1–8.

Hertel L, Rolle S, Andrea MD, Azzimonti B, Osello R, Gribaudo G, Gariglio M, Landolfo S. 2000. The retinoblastoma protein is an essential mediator that links the interferon-inducible 204 gene to cell-cycle regulation. Oncogene 19:3598–3608.

Ikonomi P, Rivera CE, Riordan M, Washington G, Schechter AN, Noguchi CT. 2000. Overexpression of GATA-2 inhibits erythroid and promotes megakaryocyte differentiation. Exp Hematol 28:1423–1431.

Inouye CJ, Seto E. 1994. Relief of YY1-induced transcriptional repression by protein-protein interaction with the nucleolar phosphoprotein B23. J Biol Chem 269:6506–6510.

Johnstone RW, Trapani JA. 1999. Transcription and Growth Regulatory Functions of the HIN-200 Family of Proteins. Mol Cell Biol 19:5833–5838.

Johnstone R W, Kerry JA, Trapani JA. 1998. the human interferon-inducible protein, IFI16, is a repressor of transcription. J Biol Chem 273:17172–17177.

Johnstone RW, Wei W, Greenway A, Trapani JA. 2000. Functional interaction between p53 and the interferon-inducible nucleoprotein IFI16. Oncogene 19:6033–6042.

Kozopas KM, Yang T, Buchan HL, Zhou P, Craig RW. 1993. *MCL1*, a gene expressed in programmed myeloid cell differentiation, has sequence similarity to BCL2. Proc Natl Acad Sci USA 90:3516–3520.

Laborda J. 2000. The role of the epidermal growth factor-like protein dlk in cell differentiation. Histol Histopathol 15:119–129.

Lee YM, Lee SC. 1994. Transcriptional activation of the alpha-1 acid glycoprotein gene by YY1 is mediated by its functional interaction with a negative transcription factor. DNA Cell Biol 13:1029–1036.

Liu CJ, Wang H, Lengyel P. 1999. The interferon-inducible nucleolar p204 protein binds the ribosomal RNA-specific UBF1 transcription factor and inhibits ribosomal RNA transcription. EMBO J 18:2845–2854.

Liu CJ, Wang H, Zhao Z, Yu S, Lu YB, Meyer J, Chatterjee G, Deschamps S, Roe BA, Lengyel P. 2000. MyoD-dependent induction during myoblast differentiation of p204, a protein also inducible by interferon. Mol Cell Biol 20:7024–7036.

Luu P, Fores O. 1997. Binding of SP1 to the immediate-early protein-responsive element of the human cytomegalovirus DNA polymerase promoter. J Virol 71:6683–6691.

Miller DA, Rosman GJ. 1989. Improved retroviral vectors for gene transfer and expression. Biotechniques 7:980–990.

Min W, Ghosh S, Lengyel P. 1996. The interferon-inducible p202 protein as a modulator of transcription: inhibition of NF-kB, c-Fos, and c-Jun activities. Mol Cell Biol 16: 359–368.

Miranda RN, Briggs RC, Shults K, Kinney MC, Jensen RA, Cousar JB. 1999. Immunocytochemical analysis of MNDA in tissue sections and sorted normal bone marrow cells documents expression only in maturing normal and neoplastic myelomonocytic cells and a subset of normal and neoplastic B lymphocytes. Hum Pathol 30:1040–1049.

Miyazato A, Ueno S, Ohmine K, Ueda M, Yoshida K, Yamashita Y, Kaneko T, Mori M, Kirito K, Toshima M, Nakamura Y, Saito K, Kano Y, Furusawa S, Ozawa K, Mano H. 2001. Identification of myeloidysplastic syndrome-specific genes by DNA microarray analysis with purified hematopoietic stem cell fraction. Blood 98:422–427.

Ohno N, Izawa A, Hattori M, Kageyama R, Sudo T. 2001. dlk inhibits stem cell factor-induced colony formation of murine hematopoietic progenitors: Hes-1-independent effect. Stem Cells 19:71–79.

Pear WS, Nolan GP, Scott ML, Baltimore D. 1993. Production of high-titer helper-free retroviruses by transient transfection. Proc Natl Acad Sci USA 90:8392–8396.

Rozzo SJ, Allard JD, Choubey D, Vyse TJ, Izui S, Peltz G, Kotzin BL. 2001. Evidence for an interferon-inducible gene, Ifi202, in the susceptibility to systemic lupus. Immunity 15:435–443.

Ruiz-Hidalgo MJ, Gubina E, Tull L, Baladron V, Laborda J. 2002. Dlk modulates mitogen-activated protein kinase signaling to allow or prevent differentiation. Exp Cell Res 274:178–188.

Seelig HP, Ehrfeld H, Renz M. 1994. Interferon-γ-inducible protein p16. A new target of antinuclear antibodies in patients with systemic lupus erythematosus. Arthritis Rheum 37:1672–1683.

Shiffman D, Mikita T, Tai JTN, Wade DP, Porter JG, Seihamer JJ, Somogy R, Liang S, Lawn RM. 2000. Large scale gene expression analysis of cholesterol-loaded macrophages. J Biol Chem 275:37324–37332.

Shimizu N, Ohta M, Fujiwara C, Sagara J, Mochizuki N, Oda T, Utiyama H. 1992. A gene coding for a zinc finger protein is induced during 12-O-tetradecanoylphorbol-13-acetate-stimulated HL-60 cell differentiation. J Biochem 111:272–277.

Takemori H, Doi J, Katoh Y, Halder SK, Lin XZ, Horike N, Hatano O, Okamoto M. 2001. Characterization of a proximal element in the rat preadipocyte factor-1 (Pref-1) gene promoter. Eur J Biochem 268:205–217.

Tannenbaum CS, Major J, Ohmori Y, Hamilton TA. 1993. A lipopolysaccharide-inducible macrophage gene (D3) is a new member of an interferon-inducible gene cluster and is selectively expressed in mononuclear phagocytes. J Leukoc Biol 53:563–568.

Wang H, Liu CJ, Lu Y, Chatterjee G, Ma XY, Eisenman RN, Lengyel P. 2000. The interferon- and differentiation-inducible p202a protein inhibits the transcriptional activity of c-myc by blocking its association with max. J Biol Chem 275:27377–27385.

Weiler SR, Gooya JM, Ortiz M, Tsai S, Collins SJ, Keller JR. 1999. D3: A gene induced during myeloid cell differentiation of Linlo c-Kit+ Sca-1+ progenitor cells. Blood 93:527–536.

Wen Y, Yan DH, Spohn B, Deng J, Lin SY, Hung MC. 2000. Tumor suppression and sensitization to tumor necrosis factor α-induced apoptosis by an interferon-inducible protein, p202, in breast cancer cells. Cancer Res 60:42–46.

Wen Y, Yan DH, Wang B, Spohn B, Ding Y, Shao R, Zou Y, Xie K, Hung MC. 2001. p202, an interferon-inducible protein, mediates multiple antitumor activities in human pancreatic cancer xenograft models. Cancer Res 61: 7142–7147.

Xie JP, Briggs JA, Olson MOJ, Sipos K, Briggs RC. 1995. The human myeloid cell nuclear differentiation antigen binds specifically to nucleolin. J Cell Biochem 59:529–536.

Xie J, Briggs JA, Morris SW, Olson MOJ, Kinney MC, Briggs RC. 1997. MNDA binds NPM/B23 and NPM-MLF1 chimera generated by the t(3;5) associated with myelodysplastic syndrome and acute myeloid leukemia. Exp Hematol 25:1111–1117.

Xie J, Briggs JA, Briggs RC. 1998. Human hematopoietic cell specific nuclear protein MNDA interacts with the multifunctional transcription factor YY1 and stimulates YY1 DNA binding. J Cell Biochem 70:489–506.

Yang TH, Tsai WH, Lee YM, Lei HY, Lai MY, Chen DS, Yeh NH, Lee SC. 1994. Purification and characteriaztion of nucleolin and its identification as a transcription repressor. Mol Cell Biol 14:6068–6074.