Transfection of Wild-Type but not Mutant *p53* Induces Early Monocytic Differentiation in HL60 Cells and Increases Their Sensitivity to Stress[1]

Debabrata Banerjee,[2] Heinz-Josef Lenz, Barbara Schnieders, David J. Manno, Jing F. Ju, C. Paul Spears, Daniel Hochhauser, Kathleen Danenberg, Peter Danenberg, and Joseph R. Bertino[3]

Molecular Pharmacology and Therapeutics Program, Memorial Sloan Kettering Cancer Center, New York, New York 10021 [D. B., B. S., D. H., J. R. B.], and Kenneth Norris Comprehensive Cancer Center, University of Southern California, Los Angeles, California 90033 [H-J. L., D. J. M., J. F. J., C. P. S., K. D., P. D.]

Abstract

HL60 cells, which lack the *p53* gene due to a deletion, were used as an *in vitro* model system to study the effect of wild-type *p53* gene expression on hematopoietic differentiation. We transfected HL60 cells with wild-type *p53* and two mutant *p53* cDNAs encoding the Val to Ala mutation at codon 143 and the Arg to Trp mutation at codon 248. Flow cytometry, growth, and cytochemical analysis for α-napthyl-butyrate esterase activity and nitroblue tetrazolium reduction indicated that wild-type *p53* but not mutant *p53* induced early monocytic differentiation in the transfected HL60 cells without terminal growth arrest. The wild-type *p53* transfectants did not differentiate along the granulocytic pathway, even when induced with 1.25% DMSO for 6 days; rather, these cells resembled monocytic cells, confirming that wild-type *p53* transfection caused these cells to become committed to differentiate along the monocytic pathway. HL60 cells transfected with wild-type *p53* were more sensitive to stress, such as growth in serum-depleted medium and exposure to a chemotherapeutic agent, etoposide.

Introduction

The tumor suppresser gene, *p53*, is the most frequently mutated gene identified in human neoplasms (1, 2). Inactivation or disruption of normal *p53* function by mutation, deletion, rearrangement, or binding to amplified gene products such as MDM2 have been described (3–5). Recent evidence suggests that *p53* acts as a tumor suppresser by preventing the propagation of DNA damage to progeny or daughter cells (6). Wild-type p53 protein enters the nucleus during DNA synthesis, transcriptionally activates other genes, and regulates the onset of DNA replication at the G_1-S boundary (7). Apart from its tumor suppresser function, it is becoming increasingly evident that *p53* participates in some manner in the processes of cellular differentiation. Transfection of wild-type p53 into K562 and L12 cells resulted in the differentiation into more mature erythroid and into a more advanced stage in the pathway of B-cell maturation, respectively (8, 9). To study the possible involvement of *p53* in cellular differentiation, we chose HL60 cells as an *in vitro* model system. There are two important advantages of the HL60 model system; (*a*) HL60 cells completely lack *p53* message and protein due to a major deletion in the *p53* gene, thereby providing a *p53* minus background (10); and (*b*) the HL60 cell line can be induced *in vitro* to differentiate into a variety of different well-characterized cell types, making it possible to study differentiation (11). In this communication, we present evidence to show that transfection of wild-type p53 into HL60 cells results in differentiation into cells of the monocytic lineage without induction of terminal growth arrest. Moreover, these wild-type *p53*-transfected cells are more sensitive to stress situations than the parental or mutant *p53*-transfected cells.

Results

Establishment of HL60 Lines Expressing Wild-Type and Mutant *p53*. Parental HL60 cells were transfected with mammalian expression vectors containing *p53* cDNA (wild-type, SN3; mutant 143, SCX3; mutant 248, 248) under control of a CMV promoter and containing the neomycin phosphotransferase gene. Single-cell initiated colonies were selected in 800 μg/ml of G-418; parental nontransfected HL60 cells did not survive this concentration of G-418. Successful transfectants were selected as "single cell clones" by serial dilution of transfected cells in 96-well plates in G-418. Wild-type *p53*-transfected cells gave rise to only three clones, while the mutant *p53*-transfected cells, as well as the parental HL60 cells transfected with pWLNeo, gave rise to more than 20 clones. Stable cell lines were established by growing three clones from each transfectant in 5-ml cultures in the presence of 800 μg/ml G-418.

The cell lines were then examined for expression of the transfected *p53* cDNA by RT-PCR (shown in Fig. 1). To prove that the transfectants contained the wild-type or mutated *p53*, the cDNAs were sequenced after amplification by PCR. The correct sequences from the wild-type, mutant 143 and mutant 248 transfectants were obtained (data not shown). Expression of the p53 protein in the transfectants was visualized by immunocytochemical staining and was quantitated by image cytometry. The levels of p53 protein expressed in the wild-type *p53* transfectants were low, and thus image analysis using software-driven videography was used to quantitate the immunostained cells. Fig. 2 shows the histograms of

Received 2/9/95; revised 8/19/95; accepted 9/1/95.
[1] This work was supported by USPHS Grant CA-08010 and National Cancer Institute Cancer Center Support Grant NCI-P30-CA-08748. D. H. is supported by an award from the V Foundation.
[2] To whom requests for reprints should be addressed, at Molecular Pharmacology & Therapeutics, Memorial Sloan Kettering Cancer Center, Box 78, 1275 York Avenue, New York, NY 10021.
[3] American Cancer Society Professor of Medicine and Pharmacology.

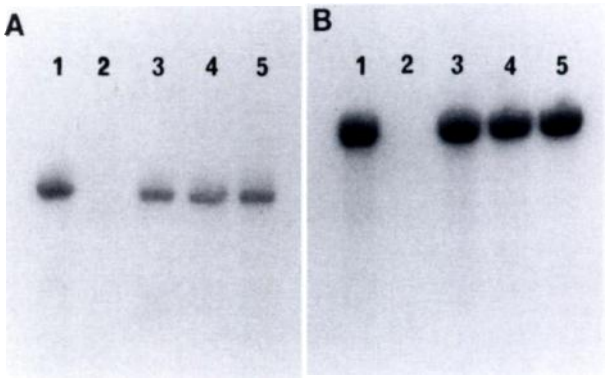

Fig. 1. Expression of *p53* visualized as T7 RNA polymerase-transcribed product from PCR amplification of reverse-transcribed *p53* cDNA from the wild-type (SN3, *Lane 3*); mutant 143 (SCX3, *Lane 4*); and mutant 248 (248, *Lane 5*) transfected HL60 cells. *A*, fragment of *p53* gene spanning exons 5 to 6; *B*, fragment spanning exons 6 to 8. *Lane 2*, complete absence of product from parental HL60 cells due to lack of expression of any *p53* message. *Lane 1*, control plasmid containing wild-type *p53* sequence, which was PCR amplified using the primer set 314/315 for exons 5 to 6 and primer set T7B/163 for exons 6 to 8.

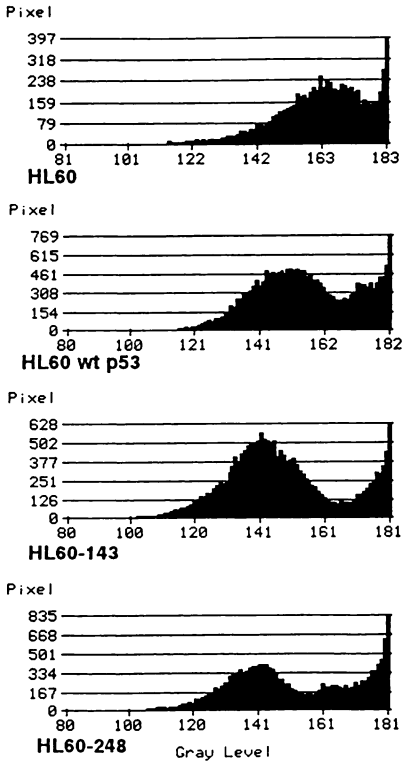

Fig. 2. Image analysis of *p53* immunostain from the parental and the three transfectants. The shift to the left of the Gray level is proportional to the intensity of *p53* immunostain. The levels of *p53* in the wild-type *p53*-transfected cells are lower than the levels in the two mutant *p53* transfectants.

intensity *versus* the number of pixels for each of the transfectants. The shift to the left of the peaks of intensity in the Gray level is indicative of positive immunostain for p53 protein. Using Raji cells that express mutant *p53* as the positive control (100%), the average levels of expression of wild-type *p53* was 1.5%, mutant 143 was 6%, and the mutant 248 was 15% in the various transfectants.

Growth Analysis. The effect of expression of *p53* (wild-type as well the mutants) on growth of HL60 cells as doubling time in liquid suspension culture and colony formation in soft agarose was evaluated. The growth of the three clones from each transfectant and the parental cells in suspension culture was measured over a period of 5 days (Fig. 3). Although transfection of wild-type *p53* did not induce terminal growth arrest of HL60 cells, all three clones of cells expressing wild-type *p53* had a marginally longer doubling time and a lower saturation density in suspension culture as compared to both the parental (pWLNeo-transfected) cells and the cells transfected with the *p53* mutant cDNAs. As the growth characteristics of the three wild-type *p53*-transfected clones were similar to each other, clone 1 from each transfectant was used for additional studies. Phase contrast microscopy of the cells in suspension culture revealed that cells transfected with wild-type *p53* grew as scattered cells, while the parental and mutant *p53*-transfected cells grew as clumps of cells both in sparse as well as dense cultures (data not shown). The colony forming ability in soft agarose of the mutant *p53*-transfected cells was comparable to that of the parental cells (average colony- forming ability in soft agarose was 25% for the mutant *p53*-transfected cells and 29% for the parental HL60 cells). The number of colonies formed by wild-type *p53*-transfected cells was consistently less (average colony-forming ability was 12%) than both parental as well mutant *p53*-transfected cells. The morphology of the colonies formed by the cells transfected with the wild-type *p53* cDNA was different from the morphology of colonies formed by the other cell lines. The colonies from parental cells and the cells transfected with the mutant *p53* cDNAs formed large compact colonies (Fig. 4; *panels 1, 3,* and *4*), while the wild-type *p53*-transfected cells formed loose colonies of scattered cells resembling colonies formed by cells that are more differentiated (Fig. 4, *panel 2*). All colonies contained clonogenic, viable cells as these could be propagated in liquid suspension culture.

Morphological and Cytochemical Characterization of the Cell Lines. To determine if wild-type or mutant *p53* cell lines showed evidence of monocytoid or granulocytic features, we examined the cell lines for morphology and enzyme activities by histochemical staining. Cytospin preparations of the four cell lines were stained with modified Wright's stain. Morphologically, the parental cells and the transfected cells (both wild-type and mutants) were indistinguishable (parental cells shown in Fig. 7*A, panel 1*; wild-type *p53* transfectants shown in Fig. 7*B, panel 1*). Cytochemical staining for α-napthyl butyrate esterase, an enzyme activity found in monocytic cells, was positive for nearly every cell (greater than 99%) transfected with wild-type *p53* cDNA (Fig. 5*A*) but was almost completely absent (less than 5%) in both parental (Fig. 5*B*) and mutant *p53* cDNA-transfected cells (data not shown). The wild-type *p53*-transfected cells also showed dense intracellular formazan deposits indicative of NBT[4] reduction in about 30% of cells as compared to

[4] The abbreviations used are: NBT, nitroblue tetrazolium; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; VP-16, etoposide; XTT, 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl-)-5-[(phenylamino) carbonyl]-2H-tetrazolium hydroxide inner salt, sodium salt.

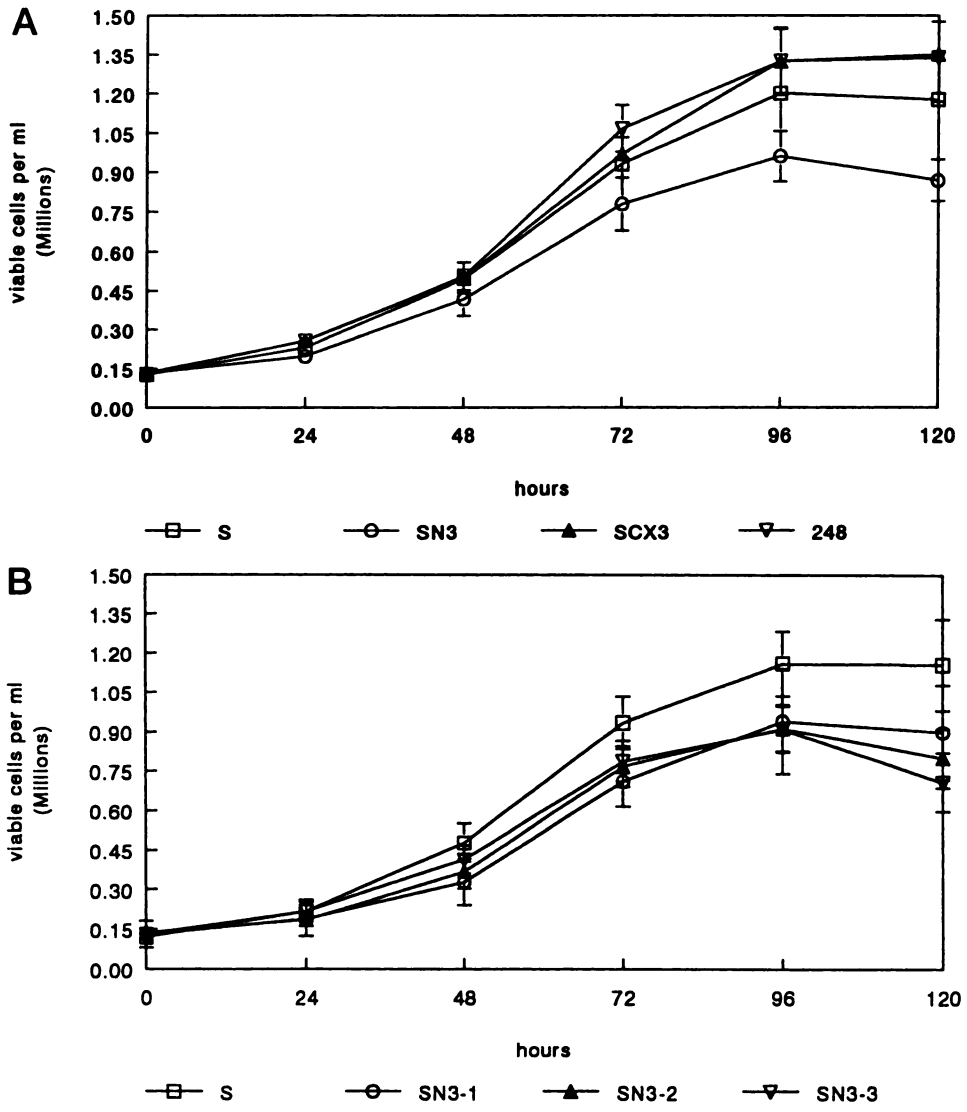

Fig. 3. A, growth in liquid suspension culture over a period of 5 days shows that the wild-type *p53* cells do not undergo growth arrest, although they have a marginally longer doubling time and a lower saturation density than the parental or the two mutant *p53*-transfected cells. Values represent the means of four independent determinations using three individual clones of each of the different transfectants; *bars,* SD. *B,* growth in liquid suspension culture for 5 days of the three individual wild-type *p53*-transfected clones as compared to the growth of the parental HL60 cells. The growth of the three clones SN3–1, -2, and -3 appear to be quite similar to each other and are quite distinct from the growth of the parental cells; *bars,* SD.

negligible NBT reduction in the parental cells as well as the cells transfected with mutant p53 cDNAs, where less than 5% of cells showed some formazan deposits (data not shown).

Surface Marker Analysis. Surface marker analyses for CD38, 34, 33, 56, 16, 15, 14, 13, and 11b were carried out by flow cytometry. All of the four cell types were positive for CD38, 33, 15, 13, and 56 but negative for CD34, 16, and 14. The HL60 cells transfected with wild-type *p53* cDNA were strongly positive (60–80%) for the CD11b surface marker (Fig. 6, *panel 2B*), while the parental cells and the cells transfected with the mutant *p53* cDNAs had the CD11b marker present in less than 5% of the cells (Fig. 6, *panels 1B, 3B,* and *4B*). The cells transfected with the mutant *p53* cDNAs did not show any change in fluorescence for the CD11b marker.

DMSO- and TPA-induced Differentiation. In the presence of DMSO, parental HL60 cells as well as the cells transfected with the two mutant *p53* cDNAs differentiated along the granulocytic pathway with an increase in banded neutrophils (shown for parental cells in Fig. 7*A, panel 3*). Initiation of morphological differentiation was apparent in about 25–30% of the parental cells treated with either DMSO or TPA. This agreed well with the increase in expression of the CD11b surface marker after addition of the differentiation inducing agents. NBT reduction increased to approximately 30% of the cell population upon induction with DMSO, suggesting that functional differentiation had taken place. There was no evidence of nonspecific esterase activity as measured by cytochemical staining for α-napthyl butyrate esterase, suggesting a lack of monocytic differentiation. The percentage of cells expressing the CD11b surface marker increased from less than 5% to about 20% for the parental and the mutant *p53*-transfected cells. There was no significant increase in the surface marker CD15 upon induction, however, as almost 80 to 90% of the uninduced cells expressed the CD15 marker; any further increase would be difficult to estimate with confidence. The CD14 surface marker was not detectable in any of the cells, even after induction with DMSO. HL60 cells transfected

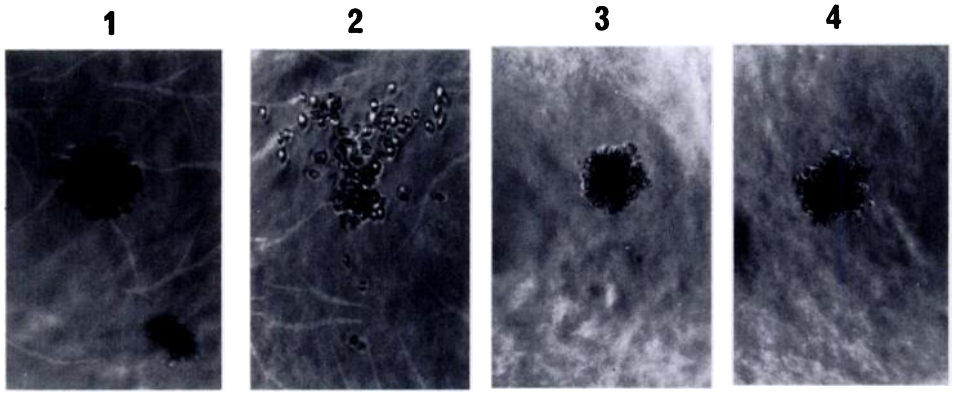

Fig. 4. Growth in soft agar. The cells from the parental (*panel 1*) and the mutant *p53*-transfected cells (mut 143, *panel 3*; mut 248, *panel 4*) appear to grow as large compact colonies in soft agar, while cells from the wild-type transfected HL60 cells grow as loose colonies of dispersed cells (*panel 2*). Data are presented for clone 1 of all four transfectants.

with the wild-type *p53* cDNA upon induction with DMSO did not show an increase in banded neutrophils; in fact, the cells appeared more monocytic than granulocytic (Fig. 7*B, panel 3*). The nonspecific esterase activity and NBT reduction remained unchanged. The CD15 (80–90%) and CD11b (60–80%) surface marker expression remained at preinduction levels. There was no detectable expression of the CD14 surface marker.

Treatment of parental as well as the mutant *p53*-transfected cells (data not shown) with TPA resulted in monocytic differentiation as evidenced by morphological characteristics (Fig. 7*A, panel 2*: parental cells). Changes in surface markers were similar to those seen for DMSO induction, except that the CD11b marker was expressed in approximately 40% of cells. Treatment of wild-type *p53*-transfected cells with 10 nM TPA also resulted in the appearance of monocytic morphological characteristics within 24 h and remained unchanged for the 6-day period (Fig. 7*B, panel 2*). Expression of the surface markers CD14 and CD15 remained unchanged from the preinduction levels, while the CD11b marker expression went up from 60–80% to greater than 90% in TPA-treated cells. There was no change in the nonspecific esterase activity and the NBT reduction in these cells (results are summarized in Table 1).

Response to Stress. HL60 cells transfected with wild-type *p53* were more sensitive to stress conditions, such as growth in serum-depleted medium and the presence of an anticancer agent such as VP-16, than the parental or the mutant *p53*-transfected HL60 cells. The results of growth in serum-depleted medium is shown in Fig. 8. All three clones of cells transfected with wild-type *p53* are unable to survive in serum-depleted medium for more than 6 days (viability was nil at the 144-h mark; *i.e.*, all cells examined took up trypan blue), while 50% or more of the parental and the mutant *p53*-transfected cells could still exclude trypan blue at the 144-h mark under the same serum-depleted conditions. Microscopic examination of stained cytospin preps of the various cells from serum-depleted medium showed that wild-type *p53* transfectants underwent apoptotic death, characterized by increased formation of cytoplasmic vacuoles and disintegration of the cell membrane, along with fragmentation of the nucleus (data not shown).

We next examined the cytotoxicity of an anticancer agent, VP-16, toward the various HL60 cell lines using a 5-day exposure and the XTT assay to measure cell survival

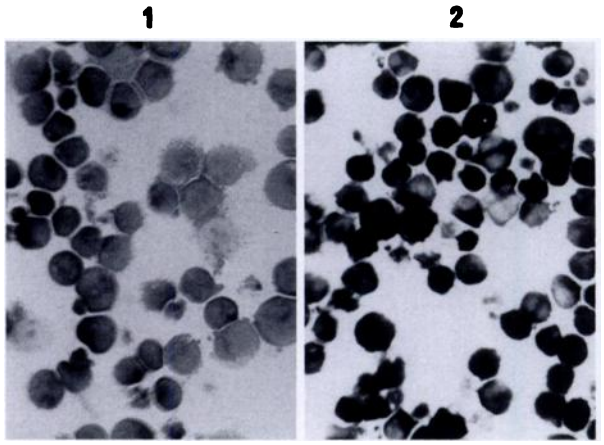

Fig. 5. Cytochemical staining for α-napthyl butyrate esterase activity. Wild-type *p53*-transfected HL60 cells (clone 1) are positive for α-napthyl butyrate esterase (*panel 2*), indicating monocytic differentiation, while the parental HL60 cells in *panel 1* are negative for the nonspecific esterase activity but take up only the methylene blue counterstain.

at the end of the drug treatment. The results are shown in Fig. 9*A*. It can be seen that the IC_{50} of VP-16 in wild-type *p53*-transfected HL60 cells is lower (2.5×10^{-8} M) compared to the parental (1.5×10^{-7} M) or the mutant 143 (9×10^{-8} M) and the mutant 248 (7×10^{-8} M) transfected cells. A separate experiment was set up where cells were treated with 10^{-7} M VP-16 for 24 h and then examined for evidence of apoptosis by microscopy as well as DNA laddering. Upon microscopic examination, it was apparent that a majority of the wild-type *p53*-transfected cells were undergoing apoptotic death. Ethidium bromide staining of the agarose gel revealed DNA laddering in wild-type *p53*-transfected cells that had been exposed to 10^{-7} M VP-16 for 24 h (shown in Fig. 9*B*).

Discussion

Transfection of wild-type *p53* cDNA into HL60 cells resulted in early changes of monocyte differentiation without inducing terminal growth arrest and without apparent morphological changes. This conclusion is supported by colony formation in soft agar, the presence of nonspecific esterase activity, and NBT reduction consistent with a more mature phenotype. Expression of the CD11b sur-

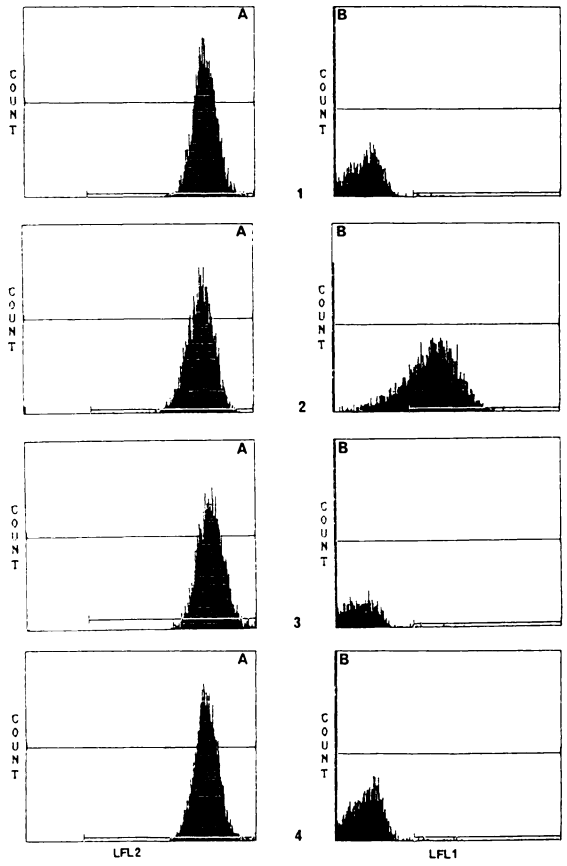

Fig. 6. Wild-type *p53*-transfected HL60 cells show increased expression of the CD11b surface marker (*panel 2B*) as compared to the parental cells (*panel 1B*) and the two mutant *p53*-transfected cells (mut 143, *panel 3B*; mut 248, *panel 4B*). The expression of CD33 surface marker for all four cell types is shown in *A*. Results for clone 1 are shown.

face marker also increased in cells transfected with wild-type *p53*. The CD11b antigen is part of the heterodimer CD11b/CD18, the C3 complement receptor, a marker of myeloid differentiation (12–19). The CD11b is not expressed to any great extent on the surface of HL60 cells but increases when HL60 cells are induced to differentiate into granulocytes, monocytes, or macrophages (15, 19). It has been shown recently by Zhang *et al.* (20) that when HL60 cells are induced to differentiate with TPA or vitamin D_3, the increase in CD11b expression is greater than when the same cells are induced with DMSO or retinoic acid, suggesting that cells committed along the monocyte/macrophage pathway express more CD11b marker than cells committed to the granulocytic pathway. The observation that HL60 cells transfected with wild-type p53 show a dramatic increase in CD11b expression is probably related to the fact that these cells are committed along the monocytic pathway. Complete differentiation of HL60 cells into mature monocytes or macrophages is accompanied by increased expression of another marker, the CD14 antigen (15, 19). The lack of CD14 expression on the surface of wild-type *p53*-transfected HL60 cells is consistent with the lack of morphological changes observed, *i.e.*, that these cells had not undergone complete differentiation.

Phorbol esters, *e.g.*, TPA, which also induce monocytic differentiation in HL60 cells, fail to induce expression of the CD14 surface marker while dramatically increasing CD11b expression (15). It has been suggested that TPA induces partial but not complete differentiation in HL60 cells (11), similar to the effect of wild-type *p53* expression as described in the present report. It is of interest that ML-1 cells, a human myeloblastic leukemia cell line, when induced to differentiate with TPA, show a dramatic increase in endogenous wild-type *p53* levels, suggesting that the primary differentiation-inducing effect of TPA may be regulated through p53 (21). However, the fact that HL60 cells, which lack endogenous *p53*, can also be induced to differentiate with TPA argue against an essential role of *p53* in hematopoietic differentiation. It should be pointed out, however, that in HL60 cells, almost 50 times more TPA is required to induce differentiation than for ML-1 cells (22). Therefore, in the absence of *p53*-induced signals, there may be an increased requirement of TPA to produce monocytic differentiation.

Soddu *et al.* (23) have reported recently that introduction of wild-type *p53* in HL60 cells induces myeloid differentiation, as indicated by an increase in expression of CD15 surface antigen accompanied by morphological changes characteristic of granulocytic differentiation. The reasons for the apparent discrepancy in the results of the present study and that reported by Soddu *et al.* (23) are not clear. The HL60 cell line that was used in the present study, when induced to differentiate with 1.25% DMSO for 6 days, shows morphological neutrophilic bands but no segmented neutrophils, in agreement with the early report of Collins *et al.* (13). However, the wild-type *p53*-transfected cell line does not differentiate along the granulocytic pathway, even in the presence of 1.25% DMSO for 6 days, suggesting that these cells are probably committed to differentiate along the monocytic pathway.

p53 is not expressed in very early hematopoietic progenitors, but as these cells differentiate into various lineage-specific cells, the expression of endogenous *p53* increases, supporting a role for *p53* in hematopoietic cell differentiation (21). Previous work on L12 and K562 cells demonstrated that transfection of wild-type *p53* induced differentiation (8, 9). In both instances, the wild-type *p53*-induced differentiation was not accompanied by terminal growth arrest. This contrasts with previous work on glioblastoma, fibroblast, and colorectal carcinoma cell lines that indicated that transfection of wild-type *p53* resulted in growth arrest (24–26). It is possible that expression of wild-type *p53* results in terminal growth arrest in cells that are already moderately differentiated but not in less differentiated cells, such as L12, K562, and HL60 cells. It should be noted that although terminal growth arrest was not observed either in L12 or K562 cells after transfection with wild-type *p53* (8, 9), expression of *p53* resulted in lower saturation densities. Moreover, as pointed out by Feinstein *et al.* (8), it is likely that successful wild-type *p53* transfectants that did not result in terminal growth arrest could be obtained only because the experimental system selects for those clones that have a very modest level of wild-type *p53* expression (8).

Response to stress was accentuated in cells expressing wild-type *p53* as compared to cells that either lacked *p53* or expressed the mutant forms of *p53*. The results suggest that reintroduction of wild-type *p53* may restore sensitivity to anticancer agents and may be therapeutically beneficial. It has been suggested that in the absence of *p53*, cells ex-

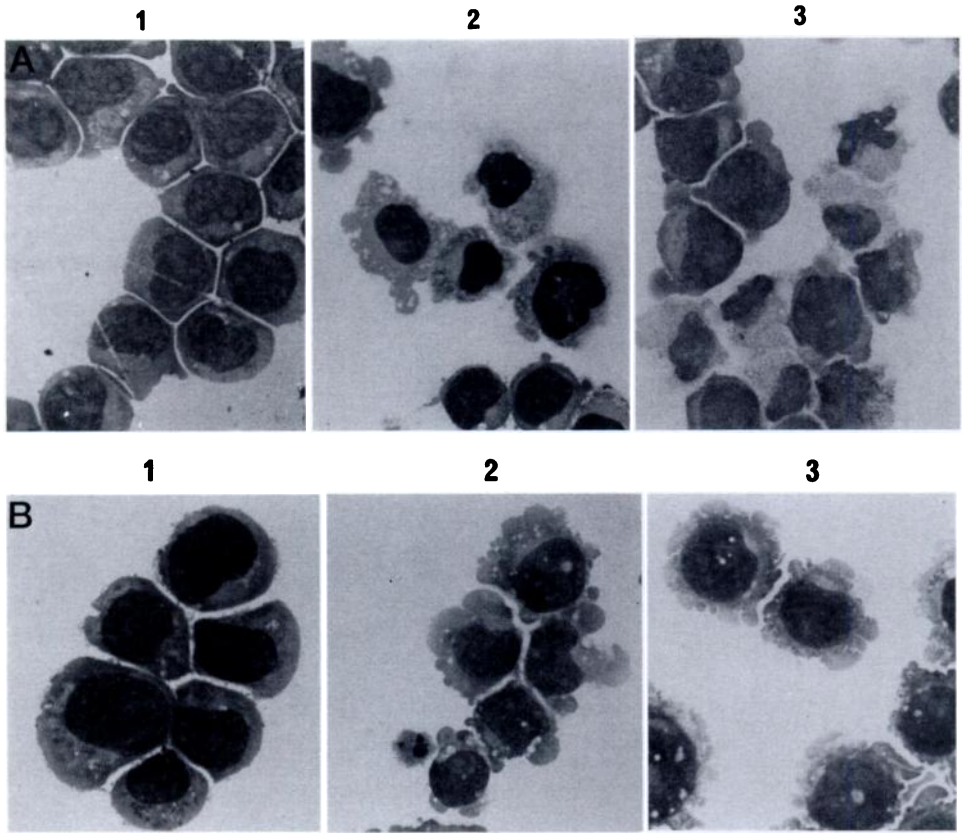

Fig. 7. *A*, parental HL60 cells (uninduced; *panel 1*) undergo morphological differentiation into monocytes when induced with 10 nM TPA (*panel 2*) and into immature granulocytes when induced with 1.25% DMSO (*panel 3*) for 6 days. *B*, wild-type *p53*-transfected cells undergo morphological differentiation only into monocytic cells when induced with either 10 nM TPA (*panel 2*) or 1.25% DMSO (*panel 3*). Data were generated using clone 1.

Table 1 Summary of induction of differentiation[a] with DMSO and TPA

Cell line[b]	Inducing agent	NSE	NBT	CD11b	CD14	CD15
HL60		–	–	–	–	+++
HL60	1.25% DMSO	–	+	+	–	+++
HL60	10nM TPA	++	+	++	–	+++
HL60-wtp53		+++	+	++	–	+++
HL60-wtp53	1.25%DMSO	+++	+	++	–	+++
HL60-wtp53 10nM TPA		+++	+	+++	–	+++

[a] +, ++, and +++, degree of activity or expression; –, absence of activity or expression.
[b] wt, wild-type.

pressing oncogenes are unable to undergo apoptosis in response to environmental stress such as chemotherapy or mitogen deprivation (27, 28). The association of *p53* with the apoptotic pathway predicts that reintroduction of wild-type *p53* function into *p53* null cells will enhance stress-induced apoptosis (29). An increase in chemosensitivity to cisplatin after introduction of wild-type *p53* was reported using adenovirus-mediated gene transfer in a human non-small cell lung cancer line H358 (30). The results may explain why tumor cells that lack *p53* or express a mutant form of *p53* are generally more resistant to stress conditions including chemotherapy. Interestingly, constitutive c-*jun* expression in U937 cells causes partial macrophage differentiation, accompanied by increased susceptibility to serum depletion. Although parental U937 cells as well as c-*myc*-transfected cells grew unabated in medium containing 0.5% serum, growth of the c-*jun*-expressing U937 cells was inhibited (31).

HL60 cells expressing wild-type as well as mutant *p53* will be of value in studying stress-induced apoptosis. Moreover, these cells provide a useful model for the study of the early changes involved in myeloid differentiation, and we are currently exploiting this system to characterize differentially expressed genes that may be important in such differentiation.

Materials and Methods

Cell Culture. HL60 cells were obtained from American Type Culture Collection (Rockville, MD) and maintained in RPMI 1640 containing 10% fetal bovine serum, 2 mM L-glutamine, and penicillin (100 units/ml) plus streptomycin (100 μg/ml). Cells in suspension culture were passaged weekly after 1:10 dilution. Transfection of wild-type and mutant *p53* cDNA containing plasmids was carried out using the *N*-[1-(2,3-dioleoyloxy)propyl]-*N*,*N*,*N*-trimethylammonium methyl sulfate (DOTAP) transfection reagent (Boehringer Mannheim, Indianapolis, IN), according to the manufacturer's instructions. The wild-type and two mutant *p53* plasmids (the 143 mutant with a valine to alanine change at amino acid residue 143; and the 248 mutant with a arginine to tryptophan change at amino acid residue 248) were obtained from Dr. Bert Vogelstein (Johns Hopkins, Baltimore, MD). As a control, the parental HL60 cells were transfected with the vector pWLNeo (Stratagene, La Jolla, CA). Cells (1×10^6) were used per transfection. Selection for successful transfectants was carried out by serial dilution in 96-well plates in media containing 800 μg/ml of G-418. The *p53* plasmids contained the neomycin phosphotransferase gene as a selectable marker. Clones of G418-resistant

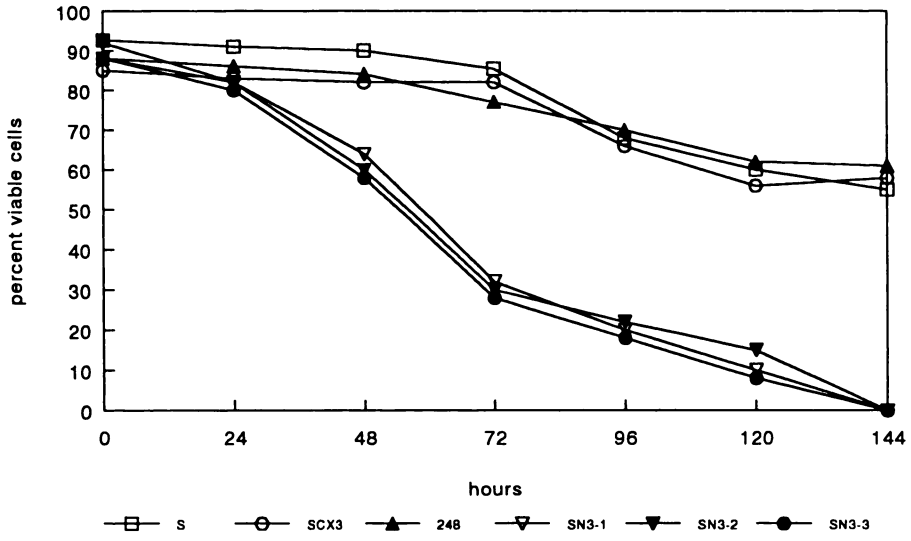

Fig. 8. Viability of cells in serum-depleted medium. HL60 cells transfected with wild-type *p53* are more sensitive to serum deprivation than the parental or the mutant *p53*-transfected cells. After 72 h of culture in serum-depleted medium, 100% of the wild-type *p53*-transfected HL60 cells took up trypan blue. The parental as well as the mutant *p53* transfectants, on the other hand, were quite resistant to serum deprivation and showed greater than 50% trypan blue-excluding cells, even after 6 days in serum-depleted medium. Data presented for parental, mutant 143, and mutant 248 transfected cells are the average of two independent experiments using clone 1 from each set and is compared with the average of two independent experiments using all three clones of the wild-type *p53*-transfected cells.

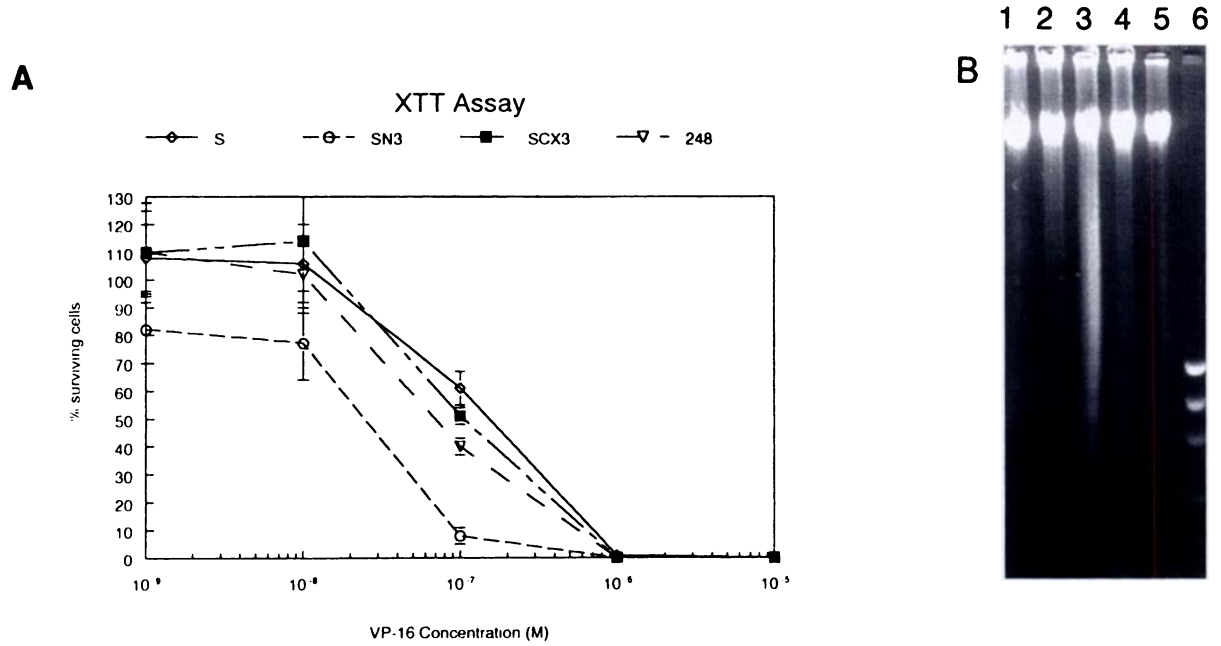

Fig. 9. *A*, Cytotoxicity of VP-16. HL60 cells transfected with wild-type *p53* are more sensitive to VP-16 than the parental or the mutant *p53*-transfected HL60 cells as measured by the XTT assay after a 5-day exposure to the drug. Data represents the means of three independent determinations using clone 1 from each transfectant; *bars*, SD. *B*, DNA fragmentation after VP-16 treatment. Wild-type *p53*-transfected cells treated with 10^{-7} M VP-16 for 24 h show characteristic apoptotic DNA laddering (*Lane 3*), which can be seen at the *bottom* of *Lane 3*, where the approximately 200-bp nucleosomal ladder is visible. The drug-treated parental (*Lane 1*) or the mutant 143-transfected (*Lane 5*) cells do not show any evidence of DNA damage. *Lanes 2* and *4*, untreated wild-type *p53* and mutant 143-transfected cells, respectively. *Lane 6* contains *X174 *Hae*III-digested DNA size markers. Genomic DNA was isolated from drug-treated and untreated cells and electrophoresed in a 1.5% TBE agarose gel for 1.5 h. At the end of the run, the gel was stained with ethidium bromide and photographed on a UV transilluminator. Clone 1 from each set of transfectants was used in this experiment.

cells obtained were expanded and then analyzed for expression of the transfected *p53* gene. RNA was isolated and reverse transcribed to cDNA from parental and transfected cells as described previously (32). The RNA was treated with DNase to eliminate the possibility of genomic DNA contamination. A fragment of the *p53* gene spanning exon 4 to exon 9 was PCR amplified using the primer set 314/334. The PCR reaction was diluted 1:20, and a second round of PCR was performed with the nested primer sets 314/315 and T7B/163. The primer set 314/315 amplifies a 215-base fragment spanning exons 5 to 6, while the primer set T7B/163 generates a 291-base fragment spanning exons 6 to 8. The cycling program consisted of 35 cycles of a denaturing step at 96°C for 60 s, an annealing step at 60°C for 60 s, and an extension step at 72°C for 60 s for the primary PCR. The secondary PCR with nested primers was

performed with the same PCR conditions, except only for 30 cycles. Each 5′ primer had a T7 promoter sequence attached to allow a T7 RNA polymerase transcription. The transcription reaction was electrophoresed on a 6% denaturing polyacrylamide gel. The dried gel was exposed to Kodak XAR5 film. Cycle sequencing of p53 cDNA was performed (according to the manufacturer's manual; GIBCO-BRL) to confirm that the correct transfected cDNA was being expressed. A fragment of the *p53* gene spanning exons 4 to 9 was amplified by PCR using the primer set 314/334. The PCR reaction was diluted 1:1000, and a second round of PCR was performed with the nested primer set 314/166. This fragment was purified using an Amicon Centricon-100 concentrator and sequenced with ^{32}P-end labeled primers 76 and 111. The cycling program consisted of 20 cycles of a denaturing step at 96°C for 60 s, an annealing step at 60°C for 60 s, and an extension step at 72°C for 60 s. A 2-μl aliquot of the sequencing reaction was electrophoresed on a 6% denaturing polyacrylamide gel. The dried gel was exposed to Kodak XAR5 film for autoradiography.

The sequences of the primers used were: p53-76, AGGGCCAGACCATCGCTAT; p53-111, ACCACCATCCACTACAACTAC; p53-163, GGTGAGGCTCCCCTTTCTTGC; p53-166, GGGCAGCTCGTGGTGAGGCTC; p53-314, CAGCCAAGTCTGTGACTTG; p53-315, TAATACGACTCACTATAGGGAGCAGCGCTCATGGTGGGGCAG; p53-334, CTGAAGGGTGAAATATTCTCCATC; and p53-T7B, GATAATACGACTCACTATAG-GGATGACAGAAACACTTT.

Growth Studies. Cell growth and viability (trypan blue exclusion) were measured over a period of 6 days. Colony formation was carried out by plating 500 cells in media containing 0.3% agarose overlayed on a basal layer of media containing 0.5% agarose. Colony number and colony morphology were examined after 12 days, and the colonies were photographed using a Zeiss photo microscope.

Morphological and Cytochemical Staining. Cytospin preparations of the parental and *p53*-transfected HL60 cells were made on a Shandon Cytospin 2 centrifuge (Shandon Southern Products, Ltd., Cheshire, United Kingdom) and stained for morphological characteristics with modified Wright's stain. Staining for α-napthyl butyrate esterase and NBT reduction were carried out on cytospin preparations of cells according to the manufacturer's instructions using reagents from Sigma Chemical Co. (St. Louis, MO). Results were documented by photography using a Zeiss photomicroscope.

Cell Surface Marker Studies. Surface antigens specific for different cell types expected from differentiating HL60 cells were determined by using fluorescently tagged mAbs specific for the various antigens on an Epics Profile FACS analyzer (Coulter Electronics, Hialeah, FL).

Induction of Differentiation with DMSO and TPA. The parental, wild-type, and mutant *p53*-transfected cells were treated with 1.25% DMSO or with 10 nM TPA and followed for 6 days for evidence of differentiation by examining cellular morphology, cell surface marker changes, and enzyme activities by histochemical staining.

Immunostaining and Image Cytometry. Immunocytochemical staining for the presence of both wild-type and mutant p53 protein in the transfectants was carried out using the monoclonal mouse anti-human p53 protein DO-7 obtained from Dako A/S (Glostrup, Denmark). Cells were air dried on slides and then stained at a dilution of 1:5. Image capture of representative fields of cells was done by use of an Optronics color CCD camera (768 × 494 pixels, 470 TVL resolution) with interlaced RGB output to a Targa+64 (Truevision) videographics framegrabber in an EISA 486/50 Mhz PC using Image Pro Plus version 2.00.06 (Media Cybernetics) as the Image analysis and operating environment. A Laborlux-S microscope with the ×40 planachromat objective and Koehler condensor adjustment of the halogen lamp were used. Background correction was done using paired stage-down defocused image files. Images were multiframe captured (30-s frames). No contrast enhancement was used. Outlines, 1-pixel width, of cells by the immunostain color selection menu were written to the bitmaps; the intensity (green) channel of the resulting composite images was extracted for area-of-interest pixel histogram measurement. The resulting histograms of intensity *versus* number of pixels clearly showed isolation of immunostain peaks and represent the results of 100 cells/image. The average SD of peaks in replicate determinations was less than 2 Gray level units. A shift to the left in Gray level is indicative of positive staining. A similar shift of the peak in a histogram of fluorescently labeled p53 antibody has been used for quantitation of very small amounts of p53 in hematopoietic cells (33).

Growth in Serum-depleted Medium and VP-16 Cytotoxicity. Cells growing in RPMI 1640 containing 10% FBS were pelleted and resuspended in RPMI 1640 without serum. Viability of cells in this serum-depleted medium was determined by trypan blue exclusion.

Cytotoxicity of VP-16 was determined by plating cells in normal growth medium and exposing them to various concentrations of VP-16 for 5 days; at the end of which, the metabolically active cell numbers were determined by the XTT assay, based on the microculture tetrazolium assay, as described previously (34). This assay measures the metabolic reduction of tetrazolium salts to water-soluble formazans by viable cells. Genomic DNA was isolated from cells treated with VP-16 (10^{-7} M, 24 h) and electrophoresed to examine VP-16-induced laddering.

References

1. Hollstein, M., Sidransky, D., Vogelstein, B., and Harris, C. C. p53 mutations in human cancers. Science (Washington DC), *253:* 49–53, 1991.

2. Levine, A. J., Momand, J., and Finlay, C. A. The *p53* tumor suppressor gene. Nature (Lond.), *351:* 453–456, 1991.

3. Nigro, J. M., Baker, S. J., Preisinger, A. C., Jessup, J. M., Hostetter, R., Cleary, K., Bigner, S. H., Davidson, N., Baylin, S., Devilee, P., Glover, T., Collins. F. S., Weston, A., Modali, R., Harris, C. C., and Vogelstein, B. Mutations in *p53* gene occur in diverse human tumors. Nature (Lond.), *342:* 705–708, 1989.

4. Mowat, M., Cheng, A., Kimura, N., Bernstein, A., and Benchimol, S. Rearrangements of the cellular *p53* gene in erythroleukemia cells transformed by Friend virus. Nature (Lond.), *358:* 633–636, 1985.

5. Oliner, J. D., Kinzler, K. W., Meltzer, P. S., George, D. L., and Vogelstein, B. Amplification of a gene encoding a p53 associated protein in human sarcomas. Nature (Lond.), *358:* 80–83, 1992.

6. Purdie, C. A., Harrison, D. J., Peter, A., Dobbie, L., White, S., Howie, S. E. M., Salter, D. M., Bird, C. C., Wyllie, A. H., Hooper, M. L., and Clarke, A. R. Tumor incidence, spectrum and ploidy in mice with a large deletion in the *p53* gene. Oncogene, *9:* 603–609, 1994.

7. Vogelstein, B., and Kinzler, K. W. p53 function and dysfunction. Cell, *70:* 523–526, 1992.

8. Feinstein, E., Gale, R. P., Reed, J., and Canaani, E. Expression of the normal *p53* gene induces differentiation in K562 cells. Oncogene, *7:* 1853–1857, 1992.

9. Shaulsky, G., Goldfinger, N., Peled, A., and Rotter, V. Involvement of wild type p53 in pre B-cell differentiation *in vitro*. Proc. Natl. Acad. Sci. USA, *88:* 8982–8986, 1991.

10. Wolf, D., and Rotter, V. Major deletions in the gene encoding the p53 tumor antigen cause lack of p53 expression in HL-60 cells. Proc. Natl. Acad. Sci. USA, *82:* 790–794, 1985.

11. Collins, S. J. The HL-60 promyelocytic leukemia cell line. Proliferation, differentiation and cellular oncogene expression. Blood, *70:* 1233–1244, 1987.

12. Breitman, T., Selonick, S., and Collins, S. J. Induction of differentiation of the human promyelocytic leukemia cell line (HL-60) by retinoic acid. Proc. Natl. Acad. Sci. USA, *77:* 2936–2940, 1980.

13. Collins, S., Ruscetti, F., Gallagher, R., and Gallo, R. Terminal differentiation of human PML cells induced by dimethylsulfoxide and other polar compounds. Proc. Natl. Acad. Sci. USA, *75:* 2458–2462, 1978.

14. Fujiki, H., Mori, M., Nakayami, M., Terada, M., Sugimura, T., and Moore, R. Indole alkaloids dihydroteleocidin B, teleocidin and lymlyatoxin A as a new class of tumor promoters. Proc. Natl. Acad. Sci. USA, *78:* 3872–3876, 1981.

15. Todd, R. F., Griffin, J. D., Ritz, J., Nadler, L. M., Abrams, T., and Schlossman, S. F. Expression of normal monocyte-macrophage differentiation antigens on HL60 promyelocytes undergoing differentiation induced by leukocyte conditioned medium or phorbol diester. Leukemia Res., *5:* 491–495, 1981.

16. Chiao, J. W., Freitag, W. F., Steinmetz, J. C., and Andreeff, M. Changes of cellular markers during differentiation of HL60 promyelocytes to macrophages as induced by T lymphocyte conditioned medium. Leukemia Res., *5:* 477–489, 1981.

17. Griffin, J. D., Ritz, J., Nadler, M., and Schlossman, S. F. Expression of myeloid differentiation antigens on normal and malignant cells. J. Clin. Invest., *68:* 932–941, 1981.

18. Griffin, J. D., Mayer, R. J., Weinstein, H. J., Rosenthal, D. S., Coral, F. S., Beveridge, R. P., and Schlossman, S. F. Surface marker analysis of acute myeloblastic leukemia: identification of differentiation associated phenotype. Blood, *62:* 557–562, 1983.

19. Todd, R. F., Nadler, L. M., and Schlossman, S. F. Antigens on human monocytes identified by monoclonal antibodies. J. Immunol., *126:* 1435–1441, 1981.

20. Zhang, W., Grasso, L., McClain, C. D., Gambel, A. N., Cha, Y., Travali, S., Deisseroth, A. B., and Mercer, W. E. p53-independent induction of *WAF1/CIP1* in human leukemia cells is correlated with growth arrest accompanying monocyte/macrophage differentiation. Cancer Res., *55:* 668–674, 1995.

21. Kastan, M. B., Radin, A. I., Kuerbitz, S. J., Onyekwere, O., Wolkow, C. A., Civin, C. I., Stone, K. D., Woo, T., Ravindranath, Y., and Craig, R. W. Levels of p53 protein increase with maturation in human hematopoietic cells. Cancer Res., *51:* 4279–4286, 1991.

22. Kazopas, K. M., Buchan, H. L., and Craig, R. W. Improved coupling between proliferation arrest and differentiation induction in ML-1 human myeloblastic leukemia cells. J. Cell. Physiol., *145:* 575–586, 1990.

23. Soddu, S., Blandino, G., Citro, G., Scardigli, R., Piaggio, G., Ferber, A., Calabretta, B., and Sacchi, A. Wild type *p53* gene expression induces granulocytic differentiation of HL60 cells. Blood, *83:* 2230–2237, 1994.

24. Mercer, W. E., Shields, M. T., Amin, M., Sauve, G. J., Apella, E., Romano, J. W., and Ullrich, S. J. Negative growth regulation in a glioblastoma tumor cell line that conditionally expresses human wild type p53. Proc. Natl. Acad. Sci. USA, *87:* 6166–6170, 1990.

25. Michalovitz, D., Halevy, D., and Oren, M. Conditional inhibition of transformation and of cell proliferation by a temperature sensitive mutant of p53. Cell, *62:* 671–680, 1990.

26. Baker, S. J., Markowitz, S., Fearon, E. R., Wilson, J. K. V., and Vogelstein, B. Suppression of human colorectal carcinoma cell growth by wild type p53. Science (Washington DC), *249:* 912–915, 1990.

27. Lowe, S. W., Ruley, H. E., Jacks, T., and Housman, D. E. p53 dependent apoptosis modulates the cytotoxicity of anticancer agents. Cell, *74:* 957–967, 1993.

28. Evan, G. I., Wyllie, A. H., Gilbert, C. S., Littlewood, T. D., Land, H., Brooks, M., Waters, C. M., Penn, L. Z., and Hancock, D. C. Induction of apoptosis in fibroblasts by c-myc protein. Cell, *69:* 119–128, 1992.

29. Lowe, S. W., Bodis, S., McClatchey, A., Remington, L., Ruley, H. E., Fisher, D. E., Housman, D. E., and Jacks, T. p53 status and the efficacy of cancer therapy *in vivo*. Science (Washington DC), *266:* 807–810, 1994.

30. Fujiwara, T., Grimm, E., Mukhopadhyay, T., Zhang, W. W., Owen-Schaub, L. B., and Roth, J. A. Induction of chemosensitivity in human lung cancer cells *in vivo* by adenovirus-mediated transfer of wild-type p53. Cancer Res., *54:* 2287–2291, 1994.

31. Szabo, E., Preis, L. H., and Birrer, M. Constitutive cJun expression induces partial macrophage differentiation in U937 cells. Cell Growth & Differ., *5:* 439–446, 1994.

32. Horikoshi, T., Danenberg, K. D., Stadlbauer, T. H. W., Volkenandt, M., Shea, L. C. C., Aigner, K., Gustavsson, B., Leichman, L., Frosing, R., Ray, M., Gibson, N. W., Spears, C. P., and Danenberg, P. V. Quantitation of thymidylate synthase, dihydrofolate reductase, and DT-diaphorase gene expression in human tumors using the polymerase chain reaction. Cancer Res., *52:* 108–116, 1992.

33. Kastan, M. B., Onyekwere, O., Sidransky, D., Vogelstein, B., and Craig, R. W. Participation of p53 protein in cellular response to DNA damage. Cancer Res., *51:* 6304–6311, 1991.

34. Seudiero, D. A., Shoemaker, R. H., Paull, K. D., Monks, A., Tierney, S., Nofziger, T. H., Currens, M. J., Seniff, D., and Boyd, M. R. Evaluation of a soluble tetrazolium/formazan assay for cell growth and drug sensitivity in culture using human and other tumor cell lines. Cancer Res., *48:* 4827–4833, 1988.