Interference with p53 Protein Inhibits Hematopoietic and Muscle Differentiation

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Abstract. The involvement of p53 protein in cell differentiation has been recently suggested by some observations made with tumor cells and the correlation found between differentiation and increased levels of p53. However, the effect of p53 on differentiation is in apparent contrast with the normal development of p53-null mice. To test directly whether p53 has a function in cell differentiation, we interfered with the endogenous wt-p53 protein of nontransformed cells of two different murine histotypes: 32D myeloid progenitors, and C2C12 myoblasts. A drastic inhibition of terminal differentiation into granulocytes or myotubes, respectively, was observed upon expression of dominant-negative p53 proteins. This inhibition did not alter the cell cycle withdrawal typical of terminal differentiation, nor $p21^{(WAF1/CIP1)}$ upregulation, indicating that interference with endogenous p53 directly affects cell differentiation, independently of the p53 activity on the cell cycle. We also found that the endogenous wt-p53 protein of C2C12 cells becomes transcriptionally active during myogenesis, and this activity is inhibited by p53 dominant-negative expression. Moreover, we found that p53 DNA-binding and transcriptional activities are both required to induce differentiation in p53-negative K562 cells. Taken together, these data strongly indicate that p53 is a regulator of cell differentiation and it exerts this role, at least in part, through its transcriptional activity.

The wt-p53 gene encodes a nuclear phosphoprotein which has many properties of tumor suppressors (Finlay et al., 1989; Baker et al., 1990; Chen et al., 1990). Mutations or inactivation of p53 are frequently associated with a large number of human cancers (Baker et al., 1989; Levine et al., 1991). Biochemical and molecular studies demonstrated that p53 is able to bind to specific DNA sequences (Kern et al., 1991) and to activate gene transcription (Farmer et al., 1992). However, the biological functions of p53 are still debated. Increased expression of endogenous p53 protein was observed during the cellular response to X-ray or drug-induced DNA damage (Kastan et al., 1991*b*; Kuerbitz et al., 1992; Lowe et al., 1993*a*,*b*; Lu and Lane, 1993). These increased levels of p53 protein seem to be responsible for an increased transcription of the $p21^{(WAF1/CIP1)}$ inhibitor of cyclin-dependent kinases and the consequent growth arrest in the G_1 phase of the cell cycle that allows DNA repair (El-Deiry et al., 1993; Dulic et al., 1994). It was also shown that wt-p53 is required for the apoptosis induced by DNA damage (Lowe et al., 1993*b*; Gottlieb et al., 1994; Morgenbesser et al., 1994), and for the maintenance of genomic stability (Livingstone et al., 1992; Yin et al., 1992). Although the functional deletion of the p53 gene, obtained by homologous recombination, showed that p53 function is dispensable for embryonic development (Donehower et al., 1992), several studies suggested that p53 plays a role in the regulation of cell differentiation (for review see Prokocimer and Rotter, 1994). A correlation between increased expression of endogenous p53 protein and some physiological differentiative processes, such as hematopoiesis (Kastan et al., 1991*a*) and spermatogenesis (Almon et al., 1993) was observed. Furthermore, overexpression of exogenous wt-p53 protein as well as cell-irradiation can partially restore differentiation of several tumor cells (Shaulsky et al., 1991; Feinstein et al., 1992; Brenner et al., 1993; Soddu et al., 1994, Aloni-Grinstein et al., 1995). However, data obtained in tumor cells cannot demonstrate a p53 role in cell differentiation (Kemp et al., 1993) since as a tumor suppressor, wt-p53 may inhibit transformation by normalizing tumor cells; the latter might re-acquire the capacity to differentiate as a mere consequence of this normalization.

The availability of dominant-negative proteins for p53 offers the possibility to directly test whether p53 has a function in cell differentiation. In this paper we interfere with the endogenous wt-p53 protein of nontransformed cells such as 32D myeloid precursors or C2C12 myoblasts, which can terminally differentiate into granulocytes and

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myotubes, respectively (Valtieri et al., 1987; Yaffe and Saxel, 1977; Blau et al., 1985). Inactivation of the endogenous p53 protein is obtained by overexpression of the dominant-negative mutants $p53Val^{135}$ or DD-p53-miniprotein (Milner and Medcalf, 1991; Shaulian et al., 1992). The morphological and biochemical analyses performed on cells induced to differentiate show a striking reduction in the ability of the transfected cells to mature into granulocytes or myotubes, demonstrating a direct p53 involvement in cell differentiation. Interference with endogenous p53 protein does not alter the typical cell cycle modifications induced by either hemopoietic or muscle differentiation processes, including $p21^{(WAF1/CIP1)}$ upregulation; this indicates that wt-p53 contributes to cell differentiation independently of its activity on the cell cycle.

We find that stimulation of C2C12 differentiation associates with a dramatic induction of endogenous p53 transcriptional activity and this activity appears to be necessary to induce cell differentiation in the p53-negative K562 cells.

This study strongly indicates that p53 is involved in hematopoietic and muscle differentiation.

Materials and Methods

Cells and Culture Conditions

32Dcl.3 murine hematopoietic precursor cells were cultured in RPMI-1640 medium (Bio-Whittaker, Inc., Walkersville, MD) supplemented with 10% heat-inactivated FBS (GIBCO BRL, Grand Island, NY), and 5% conditioned medium from the murine myelomonocytic cell line WEHI-3B as a source of crude interleukin-3 (IL-3)[1] (Lee et al., 1982). To induce granulocytic differentiation, 32D cells were cultured in RPMI-1640 with 10% conditioned medium from the human glioblastoma cell line U-87 MG as a source of crude granulocyte-colony stimulating factor (G-CSF) (Mavilio et al., 1989). This differentiation medium was replaced every 3 d.

C2C12 murine myoblast cells were cultured in DMEM (Bio-Whittaker) supplemented with 10% FBS growth medium (GM). These cells were used within passage 30 since their differentiation index (see later in this section) can decrease significantly after this passage. To avoid spontaneous differentiation the cells are not allowed to reach confluency. Muscle differentiation was induced by incubation in serum-free medium (SF) (DMEM supplemented with 10 μg/ml insulin, and 5 μg/ml transferrin), as described (Crescenzi et al., 1994). Briefly, C2C12 cells, or their transfectants, in logarithmic phase of proliferation were trypsinized and plated on Petri dishes at a concentration of $\sim 5.2 \times 10^3$ cell/cm^2. After 2–3 h at 37°C, the adherent cells were washed with PBS, and SF medium was added and replaced every 48 h. For the experiments conducted at 32°C, the cells were let to adhere at 37°C and then shifted at 32°C for 24 h before incubation in SF medium. For the experiments conducted at 39.5°C, the cells were maintained constantly at this temperature and processed as decribed for 37°C.

K562 human leukemia cells were cultured in RPMI-1640 supplemented with 10% heat-inactivated FBS.

Plasmids and Transfections

The following plasmids were used for transfection: p53cG(Val135), carrying the temperature-sensitive $p53Val^{135}$ mutant gene under the control of the HaMSV-LTR; pN53cG(Val135), obtained by cloning the selectable marker neo under the control of the RSV-LTR into the p53cG(Val135); pRSVneo, carrying the selectable marker for G418 resistance under the control of the RSV-LTR; pBabe-puro, carrying the selectable marker for puromycin resistance under the control of the SV40 late-promoter (kindly provided by H. Land, Imperial Cancer Research Fund Laboratories, London, UK); pCMV-DD, carrying the dominant-negative p53 miniprotein under the control of the CMV promoter (kindly provided by M. Oren, Weismann Institute, Rehovst, Israel); PG_{13}CAT, carrying the CAT reporter gene driven by polyoma virus promoter and 13 copies of the p53 consensus-binding sequence, or MG_{15}CAT with 15 copies of a p53 nonbinding site (Kern et al., 1992) (kindly provided by B. Vogelstein, Johns Hopkins Oneology Center, Baltimore, MD); and pMSV-val135-22/23, carrying the temperature-sensitive $p53Val^{135}$ mutant with a second, double mutation at the transacting domain which inhibits the transcriptional activity (Hinds et al., 1989; Lin et al., 1994) (kindly provided by A.J. Levine, Princeton University, Princeton, NJ).

Approximately 5×10^6 exponentially growing 32D cells were transfected by electroporation (0.2 KV, 960 μF) with a Gene Pulser (Bio-Rad Laboratories Inc., Hercules, CA). 48 h after gene transfer, cells were cloned by limiting dilution in selection medium containing 1 mg/ml G418 (GIBCO BRL) or 2 μg/ml puromycin. 32D cells were cotransfected with the expression vectors p53cG(val135) and pBabe-puro (32Dtsp53#8 and 32Dtsp53#10), or with pCMV-DD and pBabe-puro (32D-DD#2, 32D-DD#4, 32D-DD#10, and 32D-DD#13). pBabe-puro was transfected alone (32Dpuro#1) as a control.

Approximately 2×10^6 exponentially growing C2C12 cells were electroporated (0.25 KV, 960 μF), grown in mixed culture or plated at low density for clone isolation, and maintained in selection medium containing 0.75 mg/ml G418 or 3 μg/ml puromycin for three weeks. C2C12 cells were cotransfected with expression vectors p53cG(val135) and pBabe-puro (C2-tsp53#15, C2-tsp53#16, and C2-tsp53#17) or with pN53cG(Val135) (C2-tsp53 Mix). As control pBabe-puro (C2-puro#2) or pRSV-neo (C2-neo Mix) were transfected alone. C2C12 cells were also cotransfected with pBabe-puro and PG_{13}-CAT (PG-cat#6, PG-cat#15) or MG_{15}-CAT (MG-cat#3, MG-cat#7). The CAT gene integration was assessed on cell lysates by polymerase chain reaction (Soddu et al., 1994) using two primers complementary to the CAT sequence (5′-GTCAGTTGCTCAATTGTAAC-3′; 3′-GGCCGTCAAAGATGTGTATA-5′).

PG-cat#6 and PG-cat#15 cells were stably transfected, as described for the parental C2C12 cells, with pN53cG(Val135) (PG-cat#6-tsp53.8 and PG-cat#15-tsp53.7) or pRSVneo (PG-cat#6-neo.1).

K562 cells were stably transfected by electroporation with the expression vectors pN53cG(val135) (K-tsp53#7, K-tsp53#10, K-tsp53#12, K-tsp53#14), pMSV-val135-22/23 (K-ts22-23#7, K-ts22-23#8, K-ts22-23#10, K-ts22-23#12), or pRSV-neo (K-neo#1).

Viruses and Infection

The recombinant adenovirus AdJL16 (Bacchetti and Graham, 1993) carrying the human wt-p53 (kindly provided by S. Bacchetti, McMaster University, Hamilton, Ontario), and the replication-defective, E1-deleted dl312 control adenovirus (Jones and Shenk, 1979), were prepared and titrated by infection of 293 cells. Adherent C2C12 cells were infected with 20 p.f.u./cell as described (Crescenzi et al., 1995). Differentiation induction was performed as described for parental and transfected cells starting at 24-h postinfection.

Immunoprecipitation

Approximately 10^6 C2C12 cells were labeled in 3 ml of methionine-free medium containing 100 μCi/ml of [^{35}S]methionine (Dupont/New England Nuclear Italiana s.p.a. Milano, Italy) and 1% dialyzed FBS for 90 min. Cells were washed three times in cold phosphate-buffered saline (PBS) (150 mM NaCl; 50 mM NaH_2PO_4, pH 7.5) and incubated for 30 min on ice in lysis buffer (150 mM NaCl; 50 mM Tris, pH 8; 5 mM EDTA; 1% NP-40; 1 mM PMSF). The lysates were centrifuged at 100,000 *g* for 30 min and the pellets were discarded. Lysates containing equivalent amounts of proteins were subjected to immunoprecipitation by incubation for 90 min at 4°C with anti-p53 monoclonal antibodies PAb246 (Oncogene Science, Uniondale, NY) or PAb240 (kindly provided by A. Giordano, Thomas Jefferson University, Philadelphia, PA). Immunocomplexes were precipitated with ImmunopurePlus protein A (Pierce Europe B.V., The Netherlands) bound to a rabbit anti-mouse IgG antiserum (USB, Cleveland, OH), washed four times with lysis buffer, boiled for 5 min in sample buffer, analyzed on 10% SDS-PAGE, and subjected to fluorography.

Western Blotting

Approximately 5×10^6 cells were incubated for 30 min on ice in lysis buffer (20 mM Tris, pH 7.8; 50 mM NaCl; 5 mM EDTA; 1% Triton X-100; 0.2% SDS; 0.5% sodium deoxycholate; 1 mM PMSF). The lysates were centrifuged at 100,000 *g* for 30 min, and the pellets were discarded. A

1. *Abbreviations used in this paper*: G-CSF, granulocyte-colony stimulating factor; GM, growth medium; IL-3, interleukin-3; MHC, myosin heavy chain; SF, serum-free.

quantity of 50 μg of proteins per sample was separated on SDS-PAGE and blotted onto nitrocellulose membranes (Bio-Rad). After blocking nonspecific reactivity by rocking for 1 h at RT in 2% nonfat dry milk dissolved in TBST (20 mM Tris-HCl pH 7.8, 150 mM NaCl, 0.02% Tween 20), filters were probed rocking for 2 h at RT with anti-p53 PAb240 or PAb-7 (Oncogene Science) diluted in TBST. Immunoreactivity was determined using the DAB-reaction or ECL-reaction kit (Amersham Corp., Arlington Heights, IL), following the manufacturer's instructions.

Indirect Immunofluorescence

Approximately 5×10^4 C2C12 cells were plated on 35-mm Petri dishes. For $p53Val^{135}$ protein detection, cells were fixed with absolute methanol at −20°C for 30 min, rehydrated and preblocked for 30 min at RT in PBS containing 3% FBS, and incubated for 1 h at 37°C with moAb PAb122 (Boehringer-Mannheim Italia s.p.a., Milano, Italy), which recognizes a conserved, denaturation stable epitope in the COOH-terminal region of p53 protein. For myosin heavy chain (MHC) detection, cells were fixed with cold methanol:acetone (1:1) for 10 min, rehydrated, and incubated for 1 h at 37°C with moAb MF20 (Bader et al., 1982). Immunoreactions were detected by incubation with an affinity-purified, fluorescein isothiocyanate (FITC)-conjugated, goat anti-mouse-IgG $(FAb)_2$ fragment (Cappel, West Chester, PA). Nuclei were stained after immunofluorescence reaction by incubating the cells for 3 min in a 1-mg/ml solution of HOECHST 33258 dye in PBS.

Determination of Cell Differentiation

Granulocytic differentiation was assessed by morphological analysis of cytospin preparations fixed and stained with May-Grunwald Giemsa (Sigma Chem. Co., St. Louis, MO). 200 cells were counted from each sample by a light microscope.

Skeletal muscle differentiation was detected by indirect immunofluorescence for MHC. Differentiation index was calculated at the indicated time points, by counting 200 nuclei per dish, as follows:

$$\text{Differentiation index} = \frac{\text{Number of nuclei in differentiated cells}}{\text{Total number of nuclei}}$$

Erythroid differentiation was assessed by benzidine staining for the presence of hemoglobin. Benzidine stain was freshly prepared by mixing 1 ml of stock benzidine hydrochloride (Sigma) (2 mg/ml in 0.5% acetic acid) with 5 μl of 30% hydrogen peroxide. Equal volumes of benzidine stain were thoroughly mixed with cell suspensions, transferred to a hemocytometer, and cells were scored after 5 min. 400 cells were counted from each sample.

Cell Cycle Analysis, Proliferation, and Viability Curves

32D cells, 5×10^5, were fixed in cold acetone:methanol (1:4) for 30 min at 4°C, and DNA was stained with 50 μg/ml propidium iodide (Sigma) in PBS with 1 mg/ml RNAse A (Boehringer-Mannheim) for 30 min at RT. The DNA content was measured by an Epics XL analyzer (Coulter Corporation, Miami, FL). C2C12 cells, 10^5, were plated in 60-mm Petri dishes in either GM or SF. Cell numbers were determined in duplicate, at daily intervals. The means of three experiments are presented. Cell viability was determined by trypan blue exclusion test.

Northern Blot Hybridization

Total cellular RNA was extracted by the guanidium thiocyanate method (Chomczynski and Sacchi, 1987). Aliquots of 15 μg per lane were electrophoresed through 1.5% agarose gel in the presence of formaldehyde. Gels were blotted onto Duralose nitrocellulose membrane (Stratagene, La Jolla, CA) and hybridized as described (Ausubel et al., 1987). The p21 probe was a gel-purified 0.8-kb fragment of murine p21 cDNA (kindly provided by C. Schneider, Consortium for Interuniversity Biology, Padriciano 99, Italy) labeled with [^{32}P]dCTP (Dupont/New England Nuclear) by random primer extension (Ausubel et al., 1987).

CAT Analysis

Approximately 10^5 C2C12 cells were plated in 60-mm dishes and differentiation was induced by SF. Cells were lysed at the indicated times after serum withdrawal. The amount of CAT protein was evaluated by using a CAT-ELISA kit (Boehringer-Mannheim) following the manufacturer's instructions.

Results

Dominant-negative p53 Proteins Inhibit Granulocytic Differentiation of 32D Cells

To evaluate whether p53 protein is dispensable for cell differentiation of nontransformed cells, we used the dominant-negative effect of the $p53Val^{135}$ mutant (Milner and Medcalf, 1991) and the DD-p53 miniprotein (Shaulian et al., 1992) to interfere with the endogenous wt-p53 protein of 32D myeloid precursor cells. These are IL-3–dependent, diploid cells (Greenberger et al., 1983*a*, *b*), which do not spontaneously develop IL-3 independence (Askew et al., 1991), and do not induce tumors in syngenic mice (Migliaccio et al., 1989). 32D cells express a wt-p53 protein (Blandino et al., 1995) and differentiate through the granulocytic pathway in the presence of G-CSF (Valtieri et al., 1987). $p53Val^{135}$ is a temperature-sensitive protein which has a mutant configuration at 37°C but behaves like wt-p53 at 32°C (Michalovitz et al., 1990). In the mutant configuration, $p53Val^{135}$ protein has a dominant-negative effect over wt-p53 protein (Milner and Medcalf, 1991). The DD-p53 miniprotein consists of the last 89 residues of murine wt-p53, includes the oligomerization domain, and lacks the DNA-binding and -transacting domains. An antagonist effect of DD-p53 toward a coexpressed wt-p53 protein has been reported, at least partially due to the formation of functionally defective, mixed oligomers between the two proteins (Shaulian et al., 1992). We transfected 32D cells as described in Materials and Methods, and single cell clones were analyzed for dominant-negative p53 protein-expression by Western blotting (Fig. 1). Two of the strongest $p53Val^{135}$-positive clones (32Dtsp53#8 and 32Dtsp53#10), four DD-p53–positive ones (32D-DD#2,

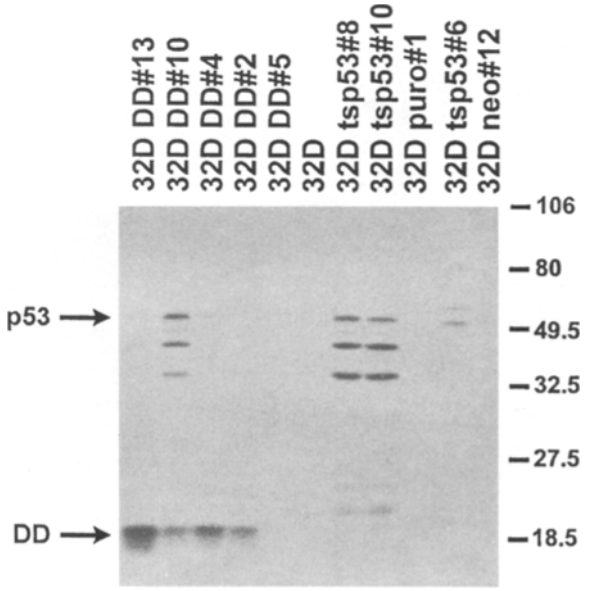

Figure 1. Biochemical analysis of transduced $p53Val^{135}$ and DD proteins. Cell lysates deriving from the indicated cells, and containing equal amounts of proteins were separated on a denaturing, 12.5% polyacrylamide gel. Western blot analysis was performed with moAb PAb240. The positions of p53 protein and DD miniprotein are indicated.

32D-DD#4, 32D-DD#10, and 32D-DD#13), and one puromycin-resistant clone (32Dpuro#1) were selected for the following experiments. The 32Dtsp53#6 and 32Dneo#12 clones, described elsewhere (Blandino et al., 1995), which carry the p53Val^{135} and neo genes or the neo gene alone, were also used. As already reported for other cell lines (Shaulian et al., 1992; Gottlieb et al., 1994), the DD-p53 transfectants expressed an increased amount of the endogenous p53 protein, probably due to its stabilization induced by the formation of wt-p53 and DD-miniprotein hetero-oligomers.

Expression of p53Val^{135} mutant at 37°C or DD-p53 miniprotein did not confer IL-3 independence to 32D cells, nor a proliferative advantage in the presence of IL-3, while they protect from apoptosis after IL-3 withdrawal (Gottlieb et al., 1994; Blandino et al., 1995; Blandino, G., and S. Soddu, unpublished results). In contrast, when the dominant-negative expressing cells were stimulated to differentiate by G-CSF, a strong inhibition of granulocytic differentiation was observed. As expected, after 8 d in the presence of G-CSF, ~80% of parental, puro- and neo-transfected 32D controls presented a decreased nuclear: cytoplasmic ratio, less basophilic cytoplasm, less prominent nucleoli, segmented and pluri-segmented nuclei, which are the typical morphological characteristics of granulocytes. In contrast, differentiation was almost absent in p53Val^{135}- and DD-p53–expressing cells (Fig. 2 *A*). This inhibition was quantified by calculating the percentages of cells in the undifferentiated, partially matured, and terminally differentiated stages of maturation (Fig. 2 *B*).

These results show that the inactivation of wt-p53 function suppresses cytokine-induced granulocytic maturation of 32D cells, thus indicating that p53 is involved in hematopoietic differentiation.

Dominant-negative p53 Inhibits Skeletal Muscle Differentiation

To evaluate whether inhibition of differentiation by interference with wt-p53 is limited to hematopoietic cells, or it is a more general phenomenon, we overexpressed p53Val^{135} mutant in a different histotype, the skeletal muscle. The myoblasts of the C2C12 murine cell line are nontransformed cells that, upon serum withdrawal, differentiate initially into mononucleated myocytes and eventually fuse into multinucleated myotubes (Yaffe and Saxel, 1977; Blau et al., 1985). We first verified the status of endogenous p53 protein by immunoprecipitation with the moAb PAb246, which reacts selectively with p53 in wild-type conformation, and with PAb240, which reacts with p53 mutant forms (Gannon et al., 1990). Only PAb246 was able to recognize p53 protein from C2C12 cell lysates, showing that C2C12 cells express an ostensibly wt-p53 protein (Fig. 3). The wild-type conformation of this p53 was also confirmed by its transcriptional activity (see below).

Clones of C2C12 cells expressing dominant-negative p53 protein were obtained by stable cotransfection with p53cG(val135) and pBabe-puro. A number of clones were screened by immunofluorescence and three of the p53Val^{135} highest expressors (C2-tsp53#15, C2-tsp53#16, and C2-tsp53#17) were selected for the following experiments, together with one puro-expressing clone (C2-puro#2) (Fig.

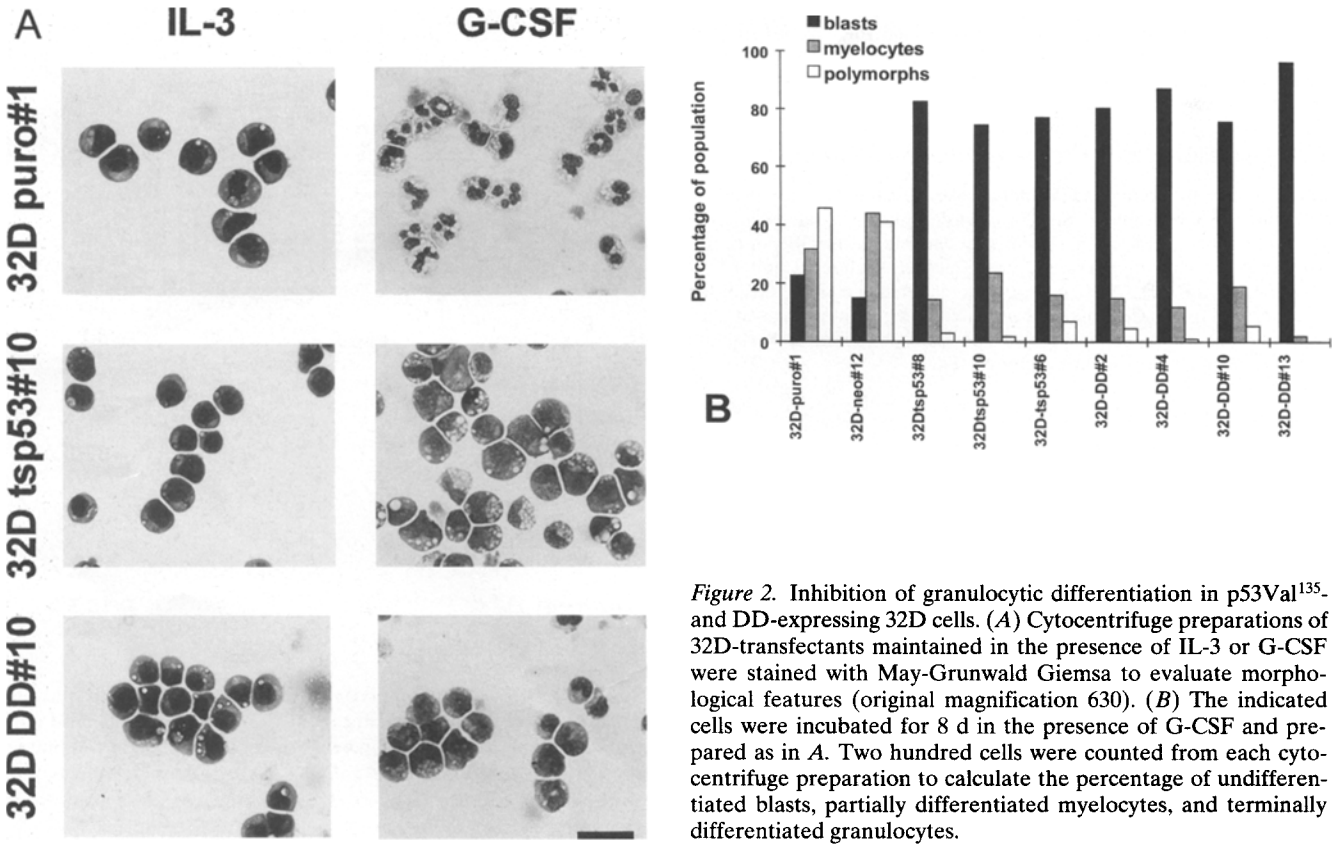

Figure 2. Inhibition of granulocytic differentiation in p53Val^{135}- and DD-expressing 32D cells. (*A*) Cytocentrifuge preparations of 32D-transfectants maintained in the presence of IL-3 or G-CSF were stained with May-Grunwald Giemsa to evaluate morphological features (original magnification 630). (*B*) The indicated cells were incubated for 8 d in the presence of G-CSF and prepared as in *A*. Two hundred cells were counted from each cytocentrifuge preparation to calculate the percentage of undifferentiated blasts, partially differentiated myelocytes, and terminally differentiated granulocytes.

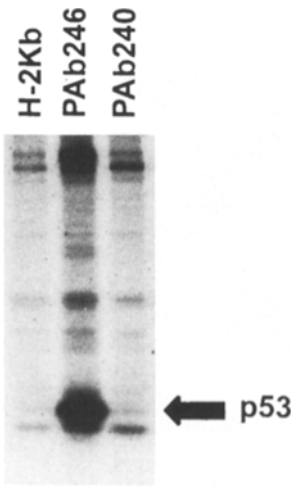

Figure 3. Immunoprecipitation of p53 from metabolically labeled C2C12 by conformation-specific moAbs. Total cell lysate was divided in three aliquots and incubated with the indicated moAbs. H-2Kb is a moAb that recognizes a major histocompatibility complex class I antigen. This antigen is not expressed on C2C12 cells. PAb246 is an anti–wild-type p53 moAb while PAb240 is an anti-mutant p53 moAb.

4, *A–D*). Since C2C12 subclones can exhibit variable abilities to differentiate, polyclonal populations were obtained by transfection of vectors encoding either the neo resistance gene and p53Val^{135} mutant or the neo resistance gene alone (C2-tsp53 Mix and C2-neo Mix). Exogenous p53 expression was detected by immunofluorescence (Fig. 4, *E* and *F*). Muscle differentiation was induced by serum starvation and morphologically differentiated, MHC-positive cells were revealed by phase contrast (not shown) and indirect immunofluorescence analyses after 72 h incubation in SF-medium (Fig. 5 *A*). A reduction of ~60% in the differentiation indices was observed in p53Val^{135}-expressing clonal and polyclonal cell populations, cultured at 37°C, compared to parental, puro- and neo-controls. This difference was maintained even after an incubation of 5 d in SF-medium (Fig. 5 *B*). Thus, interference with endogenous wt-p53 does not only impair hematopoietic differentiation, but also the differentiation of a completely different histotype such as skeletal muscle. Since p53Val^{135} partially retains wild-type activity at 37°C (Martinez et al., 1991), in an attempt to increase suppression of differentiation we repeated the experiments at 39.5°C. However, no difference was found in differentiation indices between 37°C and 39.5°C (data not shown).

C2C12 Cell Differentiation Can be Restored by Reverting the p53Val^{135} Mutant Configuration

The p53Val^{135} mutant can cooperate with activated ras in cell transformation (Michalovitz et al., 1990). Thus, we asked whether p53Val^{135} mutant expression inhibited myotube formation by inducing permanent cell modifications through genomic instability, or, alternatively, the persisting expression of dominant-negative p53 protein is necessary for suppression of differentiation. The p53Val^{135}-positive C2C12 cells were shifted to 32°C to generate wt-p53 configuration of the temperature-sensitive p53 protein, and incubated in GM or SF media. Differently from what was observed in tumor-derived cell lines (Shaulsky et al., 1991; Feinstein et al., 1992; Soddu et al., 1994), overexpression of wt-p53 alone was not sufficient to induce differentiation in the presence of serum (data not shown). However, in differentiation-promoting conditions (i.e., SF-medium)

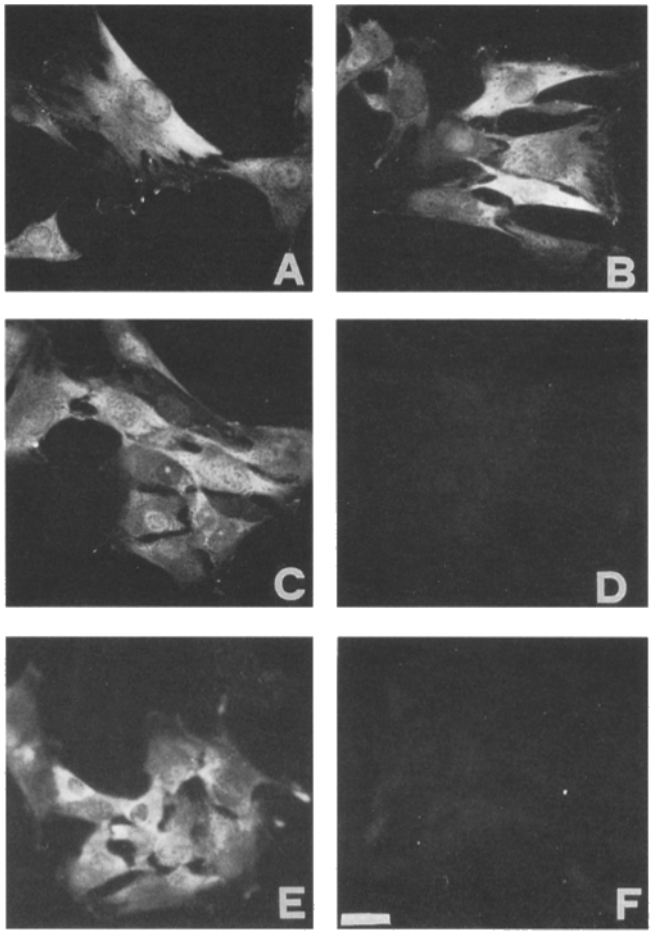

Figure 4. Exogenous p53Val^{135} mutant expression in C2C12 cells. C2-tsp53#15 (*A*), C2-tsp53#16 (*B*), C2-tsp53#17 (*C*), C2-puro#2 (*D*), C2-tsp53 Mix (*E*), and C2-neo Mix (*F*) cells were cultured in 35-mm Petri dishes, fixed, and permeabilized. p53 overexpression was detected by indirect immunofluorescence with moAb PAb122 (original magnification 400).

myotube formation was observed which, in the case of the C2-tsp53#15 and C2-tsp53#16 clones, was even increased in comparison to the control cells after 5 d of stimulation (Fig. 5 *C*). These data show that p53Val^{135}-expressing C2C12 cells do not permanently lose their ability to differentiate.

As shown in Fig. 5 *C*, incubation at 32°C reduces the differentiation index of control cells and increases the time necessary for their differentiation (not shown), probably because of a general slow down of cell metabolism. To avoid temperature and clone-to-clone variability, we infected C2C12 cells with a wt-p53–recombinant adenovirus. The E1-deleted dl312 adenovirus was used as negative control. Expression of exogenous p53 was assessed by Western blotting (data not shown). Acceleration of differentiation was observed in the Adp53-infected cells, compared to controls, whereas similar differentiation indices were reached at the 72-h endpoint (Fig. 5 *D*). Taken together, these data are suggestive of a rate-limiting function for wt-p53 protein in muscle cell differentiation, at least in the C2C12 model.

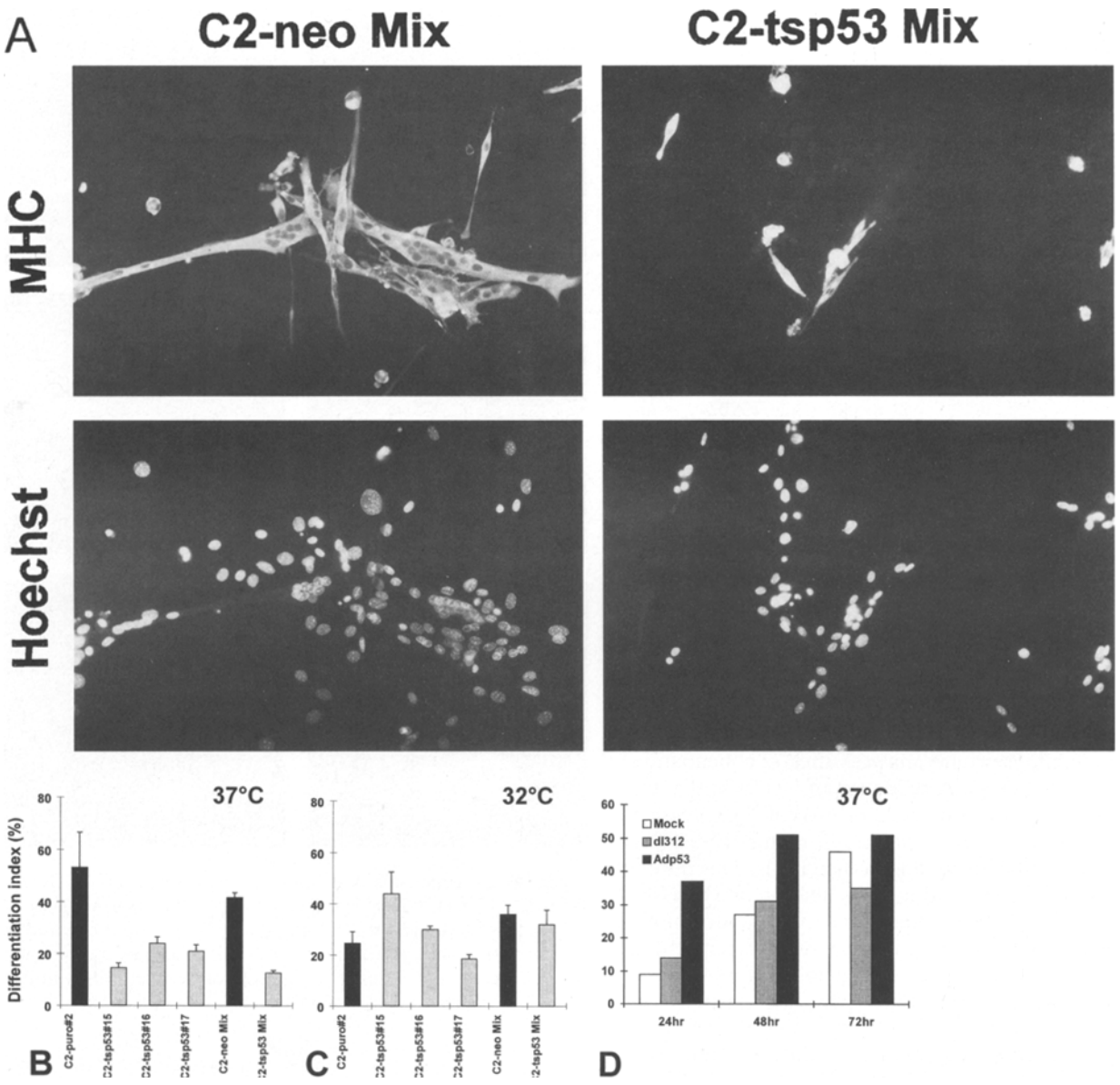

Figure 5. Inhibition of muscle differentiation in p53Val^{135}-expressing C2C12 cells. The indicated C2C12-transfectants or infected cells were induced to differentiate by serum starvation. (*A*) Myosin heavy chain (MHC) expression was detected by indirect immunofluorescence. HOECHST staining was used to count nuclei (original magnification 200). (*B*) The indicated cells were induced to differentiate at the nonpermissive temperature of 37°C. Differentiation indices were calculated as described in Materials and Methods by counting two hundred nuclei from each sample. (*C*) The indicated cells were induced to differentiate at the permissive temperature of 32°C. Differentiation indices were calculated as in Fig. 5 *B*. (*D*) Time course analysis of differentiation in C2C12 cells infected with wt-p53-recombinant, or E1-deleted dl312 adenoviruses.

Interference with or Overexpression of wt-p53 Do Not Modify Cell Viability during C2C12 Differentiation

Incubation of C2C12 cells in SF-medium to induce differentiation usually produces some cell death. Since p53 is involved in apoptotic cell death mediated by different stimuli (Lowe et al., 1993*b*; Gottlieb et al., 1994; Morgenbesser et al., 1994), it was conceivable that the changes in differentiation indices observed at 37°C and 32°C in p53Val^{135}-expressing cells was due to a variation in the total number of surviving cells, which would modify the ratio used to calculate the differentiation index (see Materials and Methods), rather than a real change in the cell capacity to undergo differentiation. To test this possibility, C2-tsp53 Mix and C2-neo Mix cells were incubated in SF-medium at 39.5°C or 32°C and cell viability was daily evaluated by trypan blue exclusion (Fig. 6, 39.5°C and 32°C). No differences in death rates were found between p53Val^{135}- and neo-expressing cells at either temperature. Similar results were obtained with the recombinant-adenovirus–infected C2C12 cells (Fig. 6, 37°C). These data show that the differences we found in differentiation indices are not due to p53-mediated alterations of death rates.

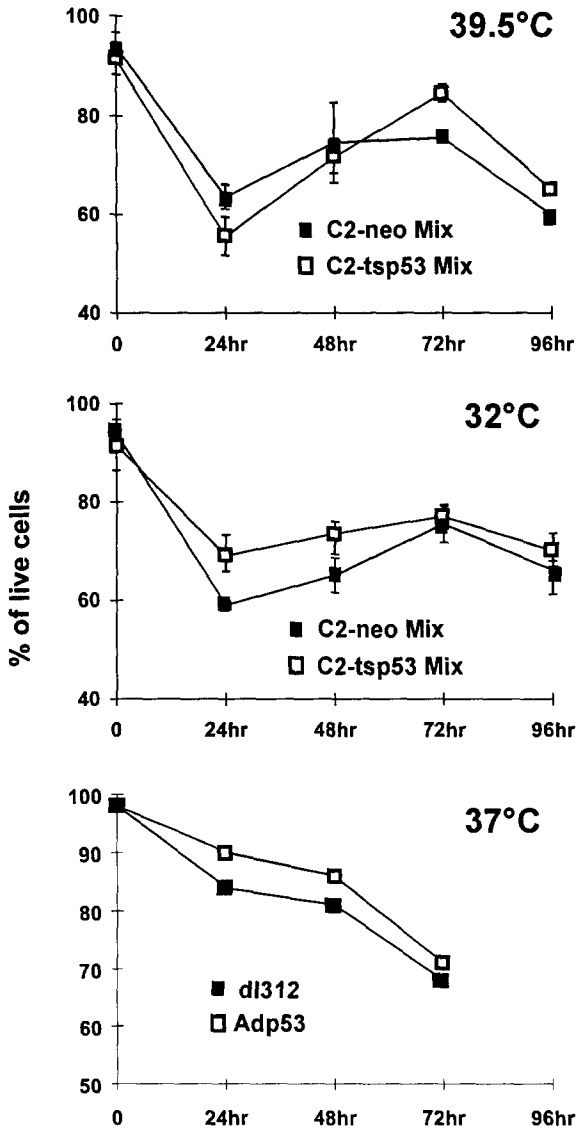

Figure 6. Viability curves of transduced C2C12 cells upon induction of differentiation. The indicated cells were stimulated to differentiate at the reported temperatures, as described in the legend to Fig. 5. Cell viability was daily evaluated by trypan blue exclusion.

Inhibition of Cell Differentiation by Interference with Wt-p53 Is Not Coupled with Stimulation of Cell Proliferation

Terminal differentiation of many cell types, such as granulocytes and myocytes, entails growth arrest (Baserga, 1985; Crescenzi et al., 1995). A number of activated oncogenes can inhibit 32D or C2C12 differentiation (Rovera et al., 1987; Mavilio et al., 1989; Kruger and Anderson, 1991; Patel et al., 1993; for review see Alemà and Tatò, 1994), at least in part through their ability to maintain the cells in a proliferative state. Moreover, wt-p53–induced growth arrest is well documented in several biological systems (Baker et al., 1990; Kuerbitz et al., 1992). Thus, we asked whether the expression of $p53Val^{135}$ mutant or DD-p53 miniprotein interfered with growth arrest in the G_0 phase

Table I. Percentages of Cells in the Different Phases of the Cell Cycle

	IL-3 Treatment			G-CSF Treatment*		
Transfectants	G_1	S	G_2/M	G_1	S	G_2/M
32D-neo#12	36.8	57.3	5.9	60.2	37.1	2.7
32D-tsp53#6	26.9	66.6	6.5	57.6	39.6	2.8
32D-tsp53#8	34.3	60.4	5.3	43.2	50.6	6.2
32D-tsp53#10	34.5	60.3	5.2	43.0	51.6	5.5
32D-puro#1	27.9	64.8	7.3	61.0	37.9	1.1
32D-DD#2	29.0	64.3	6.7	66.2	31.0	2.8
32D-DD#4	25.8	69.2	4.9	49.0	46.0	5.0
32D-DD#10	30.3	65.2	4.5	47.6	48.9	3.5
32D-DD#13	25.9	68.5	5.6	57.8	38.8	3.4

*The cells were treated with G-CSF for 8 d as indicated in Materials and Methods.

of the cell cycle, typical of cell differentiation. 32D transfectants were incubated for 8 d at 37°C in the presence of G-CSF as described above and stained with propidium iodide. DNA content was evaluated by cytofluorimetric analysis. Table I shows similar levels of accumulation in the G_0/G_1 phase of the cell cycle of $p53Val^{135}$- or DD-p53–positive and control cells. DNA content analysis is difficult to perform on multinucleated myotubes; thus, for C2C12 cells, proliferation rates were determined. As shown in Fig. 7 *A*, all cell populations similarly reduced their proliferation rates after serum withdrawal, independently of their subsequent ability to differentiate.

It has been recently shown that the expression of $p21^{(WAF1/CIP1)}$ inhibitor of cyclin-dependent kinases is induced during differentiation of hematopoietic and muscle cells, independently of p53 (Steinman et al., 1994; Halevy et al., 1995). On the basis of these observations, our dominant-negative p53-expressing C2C12 cells, which do not differentiate but stop growing, should express increased levels of p21 upon SF-stimulation. A time-course analysis of p21 transcription was performed on C2-neo Mix and C2-tsp53 Mix cells maintained in GM- or SF-medium. As expected, an upregulation of p21 starting 18 h from serum withdrawal was found in both cell populations (Fig. 7 *B*).

Taken together, these data indicate that interference with wt-p53 specifically inhibits cell differentiation without affecting the competence for withdrawal from the cell cycle.

Transcriptionally Active p53 Protein Is Induced during Muscle Differentiation

We sought to investigate whether differentiation is associated with induction of transcriptionally active p53 protein. For this purpose we stably transfected C2C12 cells with two reporter vectors carrying multiple copies of the p53 consensus-binding sequence-$PG_{13}CAT$ or of a p53 nonbinding site-$MG_{15}CAT$ (see Materials and Methods). CAT gene-positive clones were screened by polymerase chain reaction amplification (not shown) and the activity of endogenous p53 protein on the two vectors was assessed by UV-light exposure (Lu and Lane, 1993). As expected, a strong upregulation of transcription was observed only in $PG_{13}CAT$-carrying cells (data not shown). When the same clones were stimulated to differentiate into myotubes, dramatically increased CAT protein levels

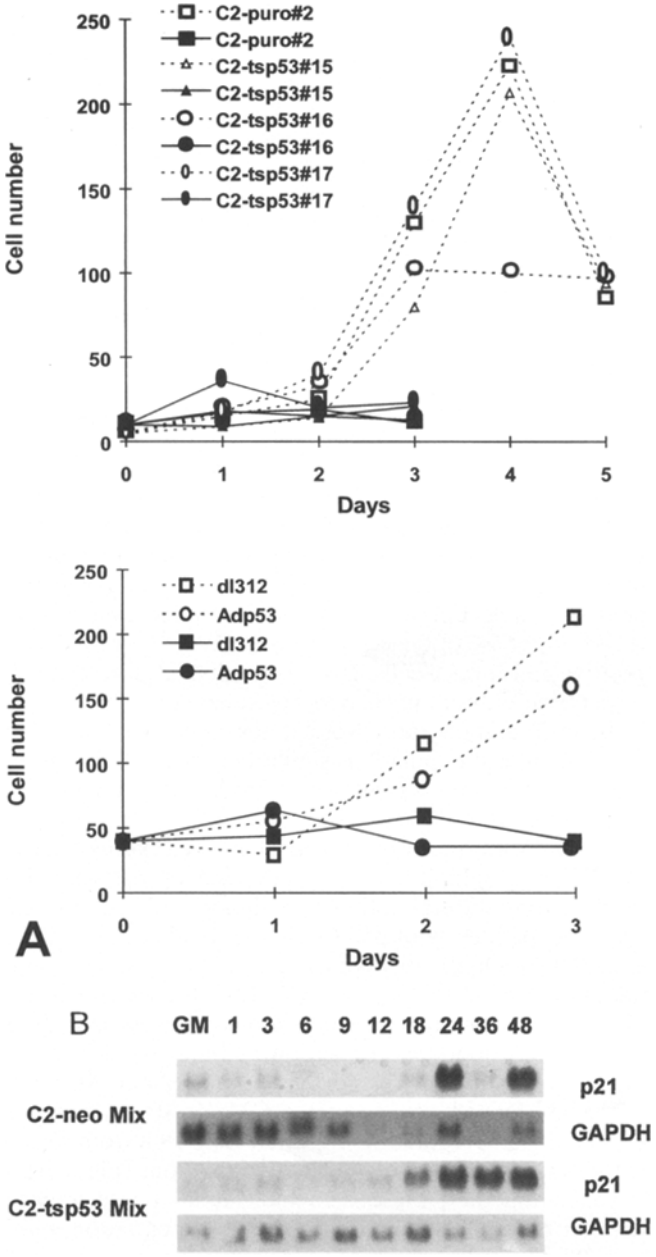

Figure 7. Growth rate analyses of transduced C2C12 cells. (*A*) The indicated cells were maintained in growing condition (GM) (*white symbols*), or in differentiation condition (SF) (*black symbols*). Cell numbers were determined in duplicate, at daily intervals, by trypan blue exclusion. Multinucleated cells were counted as single cells. The analysis in SF was stopped after 3 d because of the appearance of numerous multinucleated cells in the C2-puro#2 control. (*B*) Kinetics of $p21^{(WAF1/CIP1)}$ transcription upon SF stimulation of C2-neo Mix and C2-tsp53 Mix cells at 37°C.

were found (Fig. 8 *A*). The differences observed between PG-cat#6 and PG-cat#15 are probably due to clonal variability in differentiation kinetics. These data show that during muscle differentiation there is an increase in transcriptionally active p53 protein. This observation is in keeping with the reported upregulation of p53 mRNA during C2 muscle differentiation (Halevy et al., 1995), and it is consistent with a physiological role of p53 in cell differentiation.

To confirm that the dominant-negative mutant we used to interfere with endogenous p53 is able to suppress the wt-p53 transcriptional activity, we stably transfected PG-cat#6 and PG-cat#15 cells with the $p53Val^{135}$ mutant and determined CAT protein levels upon SF-stimulation at 37°C and 32°C. As expected, a reduction of CAT expression was observed in the $p53Val^{135}$-expressing cells, compared to neo-controls, when the experiment was performed at 37°C (Fig. 8 *B*). Opposite results were obtained at 32°C (Fig. 8 *B*).

The Transcriptional Activity of p53 Is Necessary to Induce Cell Differentiation

To evaluate whether the p53 transcriptional activity we observed in C2C12 cells during their maturation is necessary for the induction of differentiation, we compared the effects of two different p53 mutants, one in the DNA-binding domain ($p53Val^{135}$), and one in both the DNA-binding and transacting domains ($p53Val^{135}$-22/23). K562 cells, which do not express endogenous p53 protein and can be induced to differentiate along the erythroid pathway by wt-p53 overexpression (Feinstein et al., 1992), were chosen for this experiment. Cell clones were obtained by stable transfection. The expression of the two different mutants was analyzed by Western blotting (Fig. 9 *A*). Four $p53Val^{135}$- and four $p53Val^{135}$-22/23–positive clones (see Materials and Methods) were selected for the following experiments among those expressing the highest and comparable levels of the transduced proteins, together with one neo-expressing clone (K-neo#1). Differentiation induction was assessed by benzidine staining of hemoglobin-expressing cells. In agreement with the finding reported by Feinstein et al. (1992), when our K-tsp53 transfectants were shifted to 32°C to express wt-like p53 protein, spontaneous erythroid differentiation was observed (Fig. 9 *B*). In contrast, no differentiation was found in the K-ts22/23 transfectants incubated at 32°C for as long as three weeks, indicating that the DNA-binding activity of p53 is not sufficient to induce cell differentiation, and transcriptional activation is required.

Discussion

Experiments performed with tumor cells are providing increasing evidence of p53 involvement in cell differentiation (Shaulsky et al., 1991; Feinstein et al., 1992; Brenner et al., 1993; Kemp et al., 1993; Soddu et al., 1994; Pan et al., 1994). Moreover, enhanced expression of p53 protein was observed during differentiative processes as diverse as hematopoiesis and spermatogenesis (Kastan et al., 1991*a*; Almon et al., 1993). These findings are in apparent contrast with the normal development of *p53*-null mice (Donehower et al., 1992). However, targeted deletions of genes whose role in normal differentiation is well established do not overtly affect ontogenesis. This indicates that the role of important genes in normal development may escape detection due to functional redundancy. Notably, knock-outs of *MyoD* or *Myf-5* genes, that encode skeletal muscle-specific regulatory factors, generate completely normal mice,

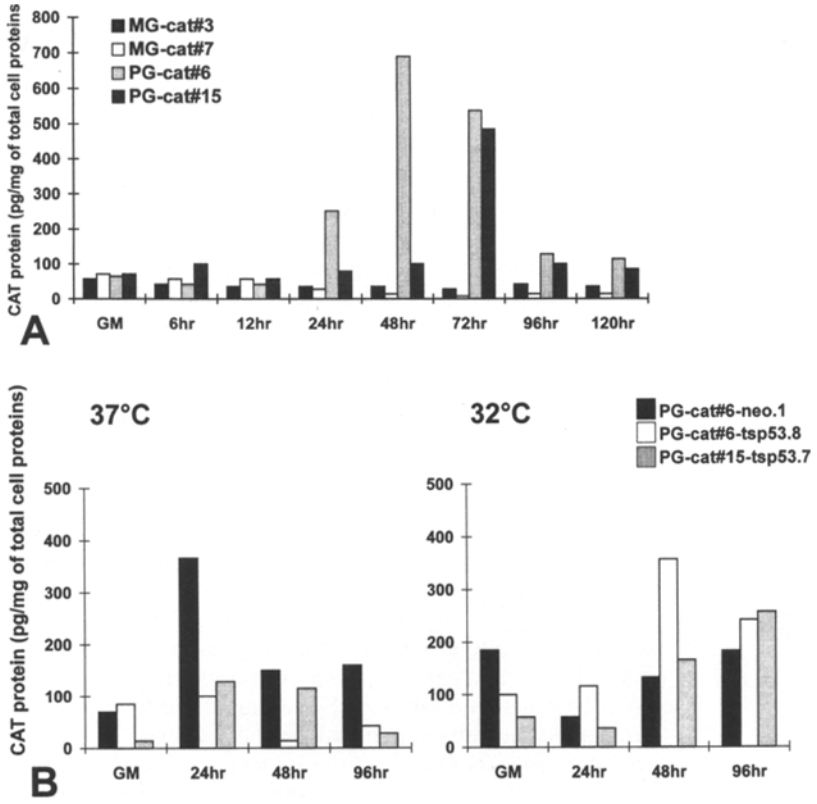

Figure 8. Time-course analysis of CAT expression. The indicated C2C12 clones were analyzed after incubation in GM or at the indicated times after serum withdrawal. (*A*) The transacting activity of endogenous wt-p53 of parental C2C12 cells is measured upon induction of differentiation. (*B*) The endogenous wt-p53 activity showed in *A* was reduced by p53Val^{135} expression in mutant configuration (37°C) and increased in wild-type configuration (32°C).

or mice with normal muscles, respectively (Rudnicki et al., 1992; Braun et al., 1992). Thus, to demonstrate that wt-p53 plays a role in physiological cell differentiation, we interfered with the endogenous wt-p53 protein of cells already determined, such as nontransformed myeloid precursor and myoblast cells. We found that expression of p53 dominant-negative proteins modified neither cell morphology nor proliferation rates, as long as the transfected cells were maintained in an undifferentiated state. However, when the transfectants were induced to differentiate, both 32D and C2C12 cells showed a striking reduction in their ability to undergo granulocyte or myotube differentiation, strongly indicating that wt-p53 is involved in cell differentiation, although this function must be somehow surrogated in *p53*-null mice.

We obtained complementary evidence in favor of a role for p53 in differentiation by overexpressing wt-p53 protein in C2C12 cells. Particularly in the p53-recombinant adenovirus-infected cells, constantly maintained at 37°C, an acceleration of myotube formation was observed after serum-starvation. Moreover, induction of C2C12 differentiation associated with increased transcriptional activity of the endogenous p53. This result is consistent with the observation made in differentiating epidermal keratinocytes in which increased p53-mediated transcriptional activity was found (Weinberg et al., 1995). We obtained data supporting the relevance of p53 transacting activity in modulating cell differentiation by overexpressing two p53 mutants into p53-negative K562 cells. We found that only wt-p53 protein induces erythroid differentiation of these cells, while the p53 mutant that conserves the DNA-binding capacity but lacks the transacting activity is no longer able to stimulate differentiation.

In both hematopoietic and muscle terminal differentiation, cells permanently withdraw from the cell cycle. Since wt-p53 can induce growth arrest in tumor cells (Baker et al., 1989; Michalovitz et al., 1990; Yin et al., 1992), we asked whether interference with endogenous wt-p53 maintained 32D and C2C12 cells in the mitotic cycle, consequently inhibiting cell differentiation. Proliferation rate evaluations showed that differentiation-promoting conditions did induce withdrawal from the cell cycle and upregulation of p21$^{(WAF\text{-}1/CIP1)}$ in dominant-negative p53-expressing as well as control cells; this indicates that inhibition of endogenous p53 interferes directly with cell differentiation independently of any effects on the cell cycle. These results are in agreement with those obtained with some leukemias in which p53-mediated maturation was not preceded by G_1 arrest (Shaulsky et al., 1991; Feinstein et al., 1992; Soddu et al., 1994). They are also in agreement with recent findings indicating that p21, a likely determinant of the growth arrest associated with muscle terminal differentiation, can be upregulated by MyoD independently of p53 (Halevy et al., 1995; Parker et al., 1995).

The functions of wt-p53 in mediating apoptotic cell death (Lowe et al., 1993*a*; Clarke et al., 1993), growth factor dependence (Yonish-Rouach et al., 1991; Gottlieb et al., 1994), and growth arrest consequent to DNA damage (Kuerbitz et al., 1992, Lowe et al., 1993*b*; Lu and Lane, 1993) are well defined. In this study we demonstrate that p53 plays a role in cell differentiation. It is possible that p53 is a cofactor in completely divergent pathways regulating all of these biological events. Alternatively, a link between these pathways can be hypothesized. Tissue and organ homeostasis are characterized, during both ontogenesis and adult life, by a well controlled balance of prolif-

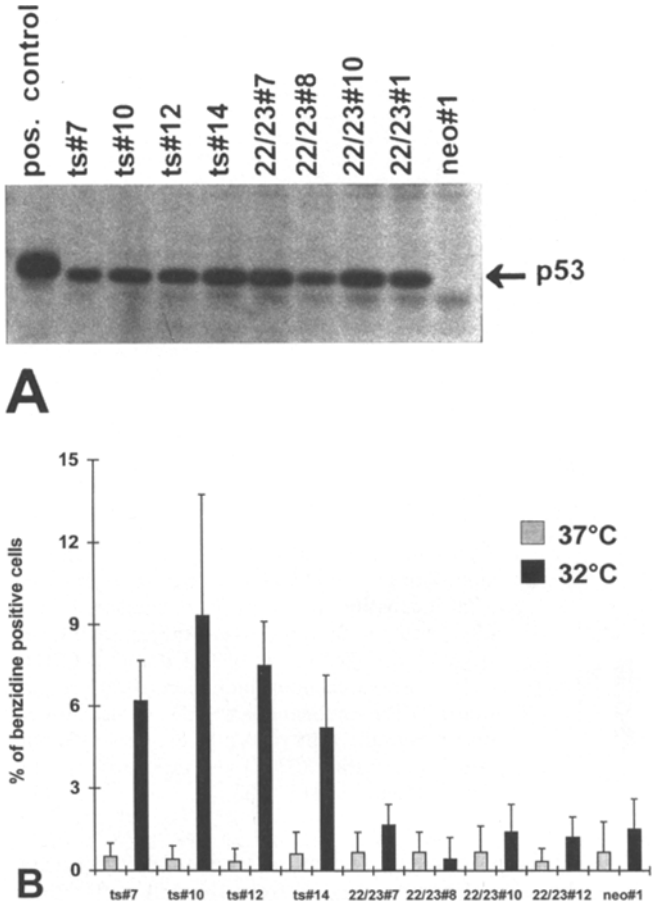

Figure 9. Induction of erythroid differentiation in K562 cells transfected with temperature-sensitive p53Val^{135} mutant or p53Val^{135}-22/23 double mutant. (*A*) Western blot analysis of exogenous p53 mutant expression. (*B*) The indicated clones were incubated at the nonpermissive (37°C) or permissive (32°C) temperature for one week. Hemoglobin was detected by benzidine reaction. Four hundred cells were counted by light microscopy to calculate the percentage of benzidine-stained cells.

eration, apoptosis, and differentiation. Hematopoiesis is a paradigmatic example of homeostasis. Stem cells can be induced to proliferate by some cytokines, and to differentiate by others. The required number of cells can be achieved by balancing apoptosis and proliferation (Dexter et al., 1977; Metcalf, 1989; Williams et al., 1990). It can be speculated that p53 acts at a cross-road linking the network of signals that regulate this homeostasis. Fig. 10 outlines a possible role for p53 in the regulation of hematopoiesis. Proliferating stem cells might receive an early differentiation signal that activates p53. Indeed, increased levels of p53 protein have been found in the course of hematopoietic maturation (Kastan et al., 1991*a*). Moreover, our data show that transiently increased levels of transcriptionally active p53 protein follow differentiation induction in C2C12 cells. Since DNA-breaks have been found during hematopoietic and muscle maturation (Johnstone and Williams, 1982; Farzaneh et al., 1982), early differentiation signals might in part function through the induction of such breaks. In other words, it is possible that p53 activity is induced through the presence of DNA-breaks, both in stress and physiological conditions. This would match the recent observation that γ-irradiation induces immunoglobulin κ light chain gene expression in pre-B lymphoid cells (Aloni-Grinstein et al., 1995). The increased levels of p53 now make the cells dependent on the presence of growth or differentiation factors. If these factors are available, the levels of active p53 decrease and the cells complete their differentiation program. In contrast, in the absence of these factors, the levels of p53 remain high and the cells die by apoptosis. This p53-mediated dependence on growth and differentiation factors was shown in M1 and DA-1 cells in which overexpression of wt-p53 induces apoptosis that can be inhibited by supplementing specific cytokines (Yonish-Rouach et al., 1991; Gottlieb et al., 1994). In addition, overexpression of wt-p53 in 32D cells has no effect as long as the cells are maintained in their normal culture conditions, but it accelerates apoptosis after interleukin-3 withdrawal, suggesting a rate-limiting effect of p53 in apoptotic cell death (Blandino et al., 1995). The fact that C2C12 cells overexpressing wt-p53 accelerate their differentiation rate only in the presence of the differentiation-promoting conditions, is also in agreement with this thesis.

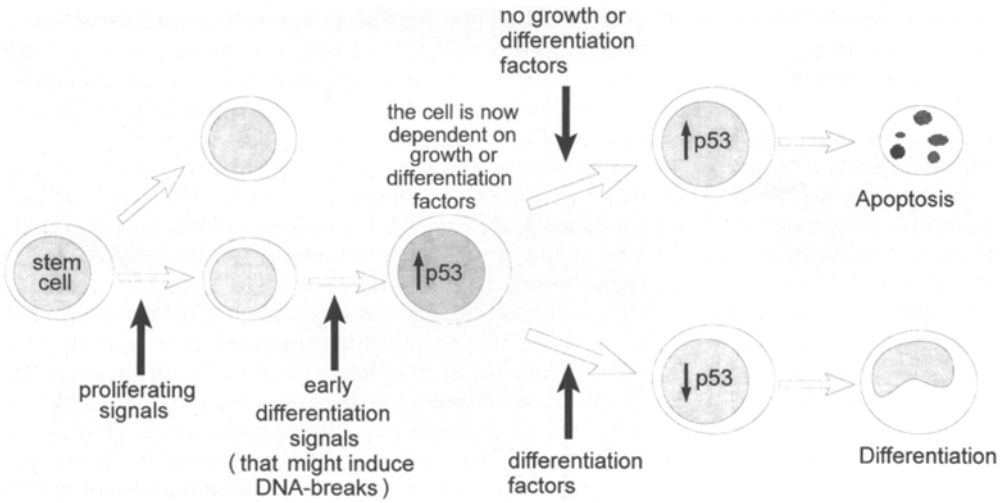

Figure 10. Model for the role of p53 in hematopoietic homeostasis. Expression of wt-p53 protein might increase during early differentiation steps. These increased levels might be induced by DNA-break formation. The cells that now express high levels of p53 become dependent on growth or differentiation factors. If these factors are available, the level of active p53 decreases and the cells can differentiate. In the absence of these factors, p53 level remains high and the cells die by apoptosis.

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