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Genetics

Involvement of wild-type p53 in pre-B-cell differentiation *in vitro*

(tumor-suppressor gene/differentiation)

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ABSTRACT **Wild-type p53 protein is a growth modulator whose inactivation has been found to be a key event in malignant transformation. Reconstitution of wild-type p53 in the p53-nonproducer, Abelson murine leukemia virus-transformed pre-B-cell line L12 gave rise to stably growing clones. Wild-type p53-producer derived cell lines exhibit an altered cell cycle, however. More cells with an extended G_0/G_1 phase were found than in the p53-nonproducer parental cell line. Furthermore, when injected into syngeneic mice, these cells induced a lower incidence of tumors and these tumors were less aggressive. Analysis of immunoglobulin expression revealed that wild-type p53 induced the expression of cytoplasmic immunoglobulin μ heavy chain. In addition, these derived cell lines exhibited increased levels of a B-cell-specific surface marker, B220. These results suggest that wild-type p53 may function as a cell differentiation factor that can induce development of pre-B cells into a more advanced stage in the pathway of B-cell maturation. In these pre-B cells, wild-type p53 may induce cell differentiation without terminal growth arrest of the cell population.**

The p53 gene, which has been shown to act as a dominant oncogene (1–4), can also function as an antioncogene (5–8). This apparent dual activity was resolved when it was found that mutant p53 expression was associated with the malignant process, whereas the wild-type p53 protein was shown to function as a negative growth regulator.

The notion that cell transformation involves the inactivation of wild-type p53 was initially deduced from the observation that the p53 gene in several types of human and mouse cell lines and primary tumors had been rearranged or inactivated by point mutations (8–14). This hypothesis was further supported by the observation that wild-type p53 failed to enhance malignant transformation (15, 16) but rather suppressed the transforming activity of other oncogenes (5, 6). A comparison of the various p53 proteins, based on their ability to transform primary rat embryonic fibroblasts in cooperation with the *ras* oncogene, indicated that mutant p53 induced the appearance of morphologically transformed foci (1–3), whereas wild-type p53 did not (15, 16). On the contrary, wild-type p53 suppressed malignant transformation of primary embryonic fibroblasts induced by cotransfection of the *ras* oncogene together with the adenovirus E1A gene, mutant p53, or *myc* (5, 6).

A more direct approach to study growth regulation by p53 was taken by Mercer *et al.* (17), who showed that the expression of wild-type p53 in human gliosarcoma cells induced growth arrest before the cells entered S phase. Conformational changes of a temperature-sensitive mutant p53 into wild-type p53 were also found to interfere with cell proliferation of transformed embryonic fibroblasts (18). These findings suggested that the activity of wild-type p53 was associated with both cell proliferation and malignancy.

In a different study (19), it was found that while transfection of wild-type p53 interfered with proliferation of a colorectal carcinoma that contained a mutated p53 gene, no effect was detectable when it was expressed in a colorectal adenoma that contained a wild-type p53 gene. It was suggested that the magnitude of wild-type p53 suppressive activity varies in cells expressing a wild-type or a mutant p53 gene (19). This notion, however, makes it difficult to estimate the net effect of p53 on cell growth regulation.

The goal of our study was to explore the physiological pathway through which p53 acts and its association with cell growth and differentiation. To that end, our strategy was to study expression of wild-type p53 in a cell system that lacks any expression of endogenous p53. In the present experiments we studied the role of wild-type p53 in pre-B-cell proliferation and differentiation. We found that introduction of wild-type p53 into cells at an early phase of their differentiation pathway induces them to advance to a more differentiated stage.

MATERIALS AND METHODS

Gene Transfer. Plasmid p53-cD codes for wild-type p53 protein (20) and the p53-M8 cDNA clone codes for a mutant p53 protein that has an alternatively spliced C terminus (20). The recombinant plasmid pSVLp53cD (20 μg) or pSVLp53M8 (20 μg) and the selective marker, pSV2gpt (5 μg; ref. 21), were introduced into exponentially growing cells (2×10^7) by electroporation (22). Clonality was verified by immunofluorescence staining against p53 and by cell morphology.

Immunoprecipitation. Cells were metabolically labeled for 1 hr at 37°C in methionine-free Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated dialyzed fetal bovine serum and [^{35}S]methionine (Amersham) at 0.125 mCi/ml (1 Ci = 37 GBq). Equal amounts of trichloroacetic acid-insoluble radioactive material were incubated for 12 hr at 4°C with specific anti-p53 antibodies PAb-240 (23), PAb-242 (24), and PAb-246 (24). To quantify newly synthesized immunoglobulin μ heavy chain, cells were metabolically labeled for 4 hr with [^{35}S]methionine before immunoprecipitation with anti-μ antibodies.

Genomic DNA Preparation and Analysis. Genomic DNA was digested either with *Eco*RI or with a combination of *Eco*RI and *Bam*HI and probed with either a 2.0-kilobase (kb) *Eco*RI–*Bam*HI insert of plasmid J_{11} (41) that hybridizes to the J_3–J_4 region of the immunoglobulin heavy-chain locus or a 0.5-kb *Pst* I insert of plasmid Bg4 (41) that hybridizes with the J_κ region of the immunoglobulin light-chain locus (probes were a gift from E. Canaani and A. Rosner, Weizmann Institute of Science).

Cell Doubling Time. Cells were plated at 2×10^5 per ml, incubated without any selectable drugs at 37°C for 24 hr, and replated at 2×10^5 per ml in fresh medium as above.

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Abbreviation: Ab-MuLV, Abelson murine leukemia virus.

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Twenty-four hours later, growth parameters of the cultured cells were examined. The number of viable cells was determined at 12- to 18-hr intervals after plating by staining triplicate samples with 0.05% eosin Y in phosphate-buffered saline (PBS). In all cases, cell viability was >98%.

Cell Cycle Analysis. Exponentially growing cells were washed twice with PBS, fixed with precooled (−20°C) 70% ethanol at 0°C for 30 min, and resuspended in PBS containing 0.5% Tween 20. The cells were stained with a fluorescein-conjugated mouse anti-BrdUrd antibody (Becton Dickinson no. 7583) and resuspended in PBS containing propidium iodide (Sigma) at 50 μg/ml. At least 5×10^3 cells (as gated by light scatter) were analyzed with a FACScan (Becton Dickinson) flow cytometer using the Consort 30 program. DNA histograms indicating linear propidium iodide fluorescence intensity were generated.

Detection of Cytoplasmic Immunoglobulin μ Heavy Chain. Cells were permeabilized and fixed with precooled (−20°C) 70% ethanol for 30 min at 0°C and stained for 30 min at 0°C with a 1:10 dilution of fluorescein-conjugated goat anti-mouse μ-chain antibody (Sigma). Cells were washed and then were resuspended in PBS containing propidium iodide at 50 μg/ml. At least 5×10^3 cells were analyzed.

Expression of the B220 Cell Surface Marker. Rat anti-mouse B220 antibody (25) (B220 clone 14.8, American Type Culture Collection) was used for flow cytometric analysis as above.

Tumorigenicity in Mice. L12-derived clones, from the earliest passage available, were grown in RPMI-1640 medium without the selective drugs for 48 hr prior to injection. Cells (2×10^6) were injected subcutaneously into individual syngeneic, male C57L/J mice. The mice were monitored daily for tumor development and were graded from 0 to 5, according to tumor size and the overall clinical status. The tumorigenicity index was calculated as described (26).

RESULTS

Establishment of L12 Cell Lines Expressing Wild-Type p53. To elucidate the mechanism of action of wild-type p53 and to assess the significance of its expression *in vivo*, we studied the expression of wild-type p53 in the p53-nonproducer cell line L12. This cell line, which is an Abelson murine leukemia virus (Ab-MuLV)-transformed lymphoid pre-B-cell line, was the first example of a p53-nonproducer line (4, 27). The p53 gene of these cells has been rearranged by integration of a Moloney murine leukemia virus into the first p53 intron (4, 27). Whereas Ab-MuLV-transformed p53-producer cells develop lethal tumors in syngeneic mice, L12 cells develop regressor tumors. Reconstitution of mutant p53 in L12 cells changed their phenotype from regressor tumors to lethal tumors (4, 27). This led to the conclusion that mutant p53 enhanced the transformed phenotype of these p53-nonproducer cells.

To establish wild-type p53-producer cell lines, L12 cells were cotransfected with the murine wild-type p53 (20) and the selectable bacterial *gpt* gene. Drug-resistant clones obtained 2–3 weeks after gene transfer were single-cell cloned and the resulting L12-cD established cell lines were further analyzed.

The p53 protein expressed in these clones was evaluated by its specific binding to anti-p53 monoclonal antibodies. Mutant p53 proteins can be distinguished from the wild-type p53 protein by their differential expression of specific antigenic epitopes. While mutant p53 forms retained the antigenic epitope recognized by monoclonal antibody PAb-240, wild-type p53 lacked this epitope and bound the anti-p53 monoclonal antibody PAb-246 instead (23, 24). Fig. 1 shows that in all L12-cD-derived clones, the expressed p53 protein bound PAb-246 specifically (lanes d); no immunoprecipitation was evident when the mutant-specific PAb-240 was used (lanes b). When 230-23-8, a p53-producer Ab-MuLV-established

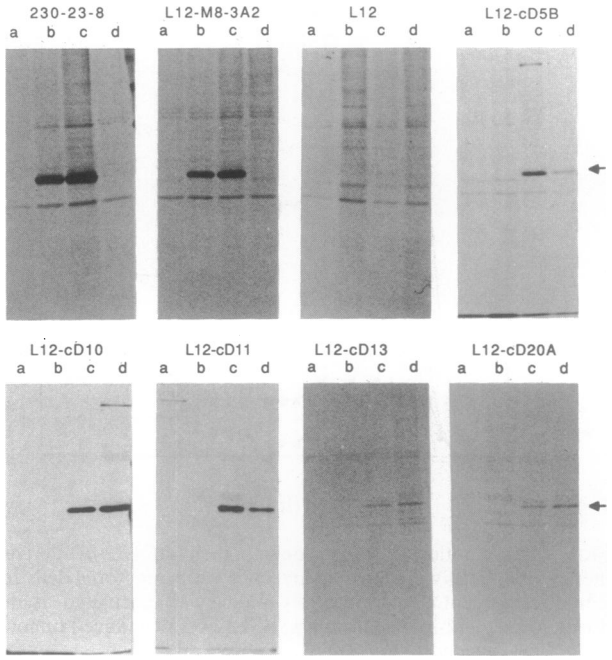

FIG. 1. Expression of wild-type p53 protein in L12-cD-derived clones. Equal amounts of trichloroacetic acid-insoluble radioactive protein were subjected to immunoprecipitation with nonimmune serum (lanes a) or with antibody PAb-240 (lanes b), PAb-242 (lanes c), or PAb-246 (lanes d). Arrows point to the position of p53 protein.

cell line, or L12-M8-3A2, an L12-derived cell line established by transfection of mutant p53 cDNA, was tested, we found, as expected, that the protein specifically immunoprecipitated with PAb-240 (lanes b), but no binding was evident when PAb-246 (lanes d) was used instead. Wild-type and mutant p53 expressed the PAb-242 antigenic determinant (lanes c). In agreement with previous studies (4, 27), L12 totally lacked p53 expression (Fig. 1). These results strongly suggest that the protein expressed in the L12-derived cell lines was the authentic wild-type p53 protein that is expected of the p53-cD cDNA clone.

These cell lines represent an exceptional example of transformed cells that proliferate in spite of constitutive expression of wild-type p53. However, since it had been shown that p53 induces cell growth arrest in several experimental systems (17–19), we examined whether these L12-derived clones exhibited any variations in their growth rate when compared with their parental cell line.

***In Vitro* and *in Vivo* Growth Patterns of L12-Derived Cell Lines.** To evaluate the effect of wild-type p53 expression on the capacity of L12 cells to grow *in vivo*, we measured the rate of tumor development in mice. The various lines were injected into syngeneic mice and tumor development was monitored. Tumor development of the L12-cD-derived clones was reduced compared with the parental L12 cells. Expression of wild-type p53 caused a reduction in the incidence and size of the developing tumors. The most striking results were obtained when L12-cD5B and L12-cD10 were compared with L12 parental cells (Fig. 2).

To evaluate differences in *in vitro* cell proliferation, we measured the doubling times of the various cell lines. To estimate the doubling time, the viable cells of growing cell lines were counted at various time points. We found that while L12 or L12 expressing mutant p53 (L12-M8-3A2) had almost identical doubling times, L12-cD-derived clones expressing wild-type p53 exhibited an increment of up to 60% in the doubling time (see L12-cD10, Table 1). Prolongation of doubling time was directly correlated with the levels of p53

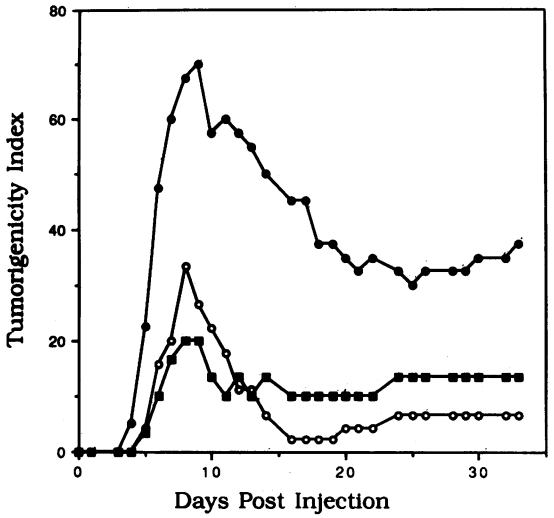

FIG. 2. Evaluation of tumor development of L12-cD-derived clones *in vivo*. Cells were injected and mice were monitored daily for tumor development. Tumorigenicity index was calculated as described (26). ●, L12-induced tumors; ○, L12-cD5B-induced tumors; ■, L12-cD10-induced tumors.

synthesis. At each time point, viability of cells was close to 100% and no morphological changes were observed.

To gain more insight into the effect of wild-type p53 on the cell cycle of L12 cells, we determined the distribution of DNA content in the population by flow cytometry. We found that all L12-cD cell lines consistently contained a significantly higher percentage of cells in the G_0/G_1 phase (up to 14%) than the L12 cells (Table 1). The cell cycle pattern of the L12 cell line is rather unusual in that the ratio of cells G_0/G_1 to those in S phase is low (Fig. 3). However, L12-cD5B and L12-cD10 presented a typical cell distribution pattern with respect to cell cycle phases (Fig. 3). L12-derived cell lines established by transfection either with mutant p53 encoded by pSVLp53M8 (L12-M8-2C1 and L12-M8-3A2) or with the drug-resistance *gpt* gene only (L12-gpt-1A3) have shown no significant changes in their cell cycle patterns compared with the parental L12 cell line (Table 1). This suggests that alterations in the cell cycle patterns observed in L12-cD-derived cell lines are most likely a result of expression of wild-type p53 protein rather than clonal selection.

Pre-B-Cell Differentiation. The L12 pre-B-cell line was established by infection of bone marrow cells with Ab-MuLV (28). An analysis of pre-B-cell lines established by Ab-MuLV showed variable levels and different species of immunoglobulin synthesis. However, ≈40% of these cell lines produced

Table 1. Distribution of cells in the various phases of the cell cycle

Cell line	Doubling time, hr	% cells		
		G_0/G_1	S	M/G_2
L12	10.6 ± 0.7	33.82	32.95	33.23
L12-cD10*	16.8 ± 0.4	47.81	28.89	23.30
L12-cD5B*	14.6 ± 0.6	45.60	27.40	27.00
L12-cD11*	14.0 ± 0.3	—	—	—
L12-cD20A*	13.8 ± 0.5	—	—	—
L12-M8-2C1†	10.2 ± 0.2	38.86	34.20	26.94
L12-M8-3A2†	9.8 ± 0.8	34.55	38.06	27.39
L12-gpt-1A3‡	10.6 ± 0.5	38.85	33.72	27.43

Cell cycle was analyzed as described in experimental procedures.
*L12 cell transfected with p53cD and pSVgpt.
†L12 cell transfected with p53-M8 and pSVgpt.
‡L12 cell transfected with pSVgpt only.

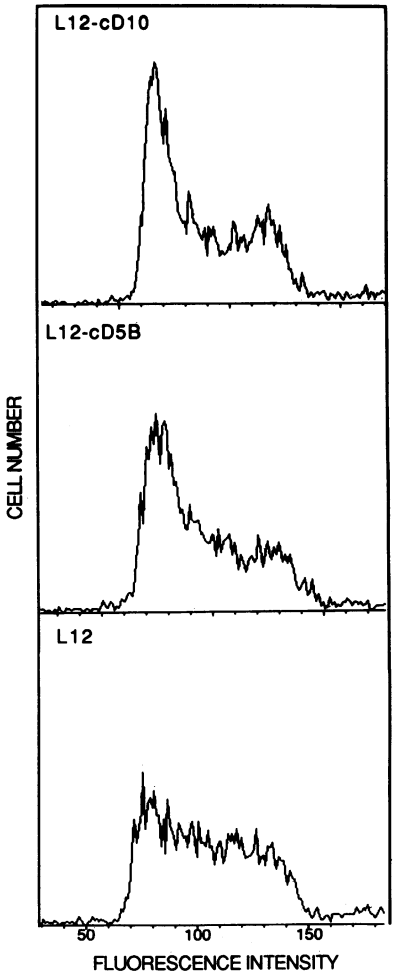

FIG. 3. Effect of wild-type p53 on L12 cell cycle. Shown are histograms indicating propidium iodide fluorescence intensity of L12, L12-cD5B, and L12-cD10. On the linear horizontal axis scale, 70 represents 2*N* chromosomes and 140 represents 4*N* chromosomes.

no immunoglobulin molecules (29). DNA from the vast majority of Ab-MuLV-transformed cell lines, regardless of whether μ-positive or immunoglobulin-null cell lines, exhibited rearrangements in both heavy-chain alleles (42). Some Ab-MuLV-transformed cells, however, were shown to differentiate *in vitro* to a more advanced stage, involving rearrangements in the immunoglobulin light-chain gene leading to the synthesis of κ light chain (30).

To evaluate possible changes in cell differentiation stage of L12-cD-derived cells expressing the wild-type p53, we analyzed the expression of immunoglobulin proteins as well as organization of the immunoglobulin heavy- and light-chain genes. To that end, cells were metabolically labeled and cell lysates were immunoprecipitated with anti-μ antibodies. Whereas no μ chain was detected in L12 parental cells, L12-cD5B and L12-cD10 exhibited significantly increased levels of μ synthesis (Fig. 4*A*). The levels of μ synthesis, which varied in the five individual L12-cD cell lines, were found to be collinear with the levels of p53 synthesis (data not shown). Analysis of the L12-M8-3A2 cell line expressing a mutant p53 protein did not reveal any significant immunoglobulin μ-chain synthesis (Fig. 4*A*).

On Southern blot analysis, the parental L12 pre-B cells exhibited rearrangements in both heavy-chain alleles. The typical 6.2-kb *Eco*RI fragment found in the germ line (30) was not evident in L12 cells; instead, smaller fragments were detected. However, no further rearrangements were found in

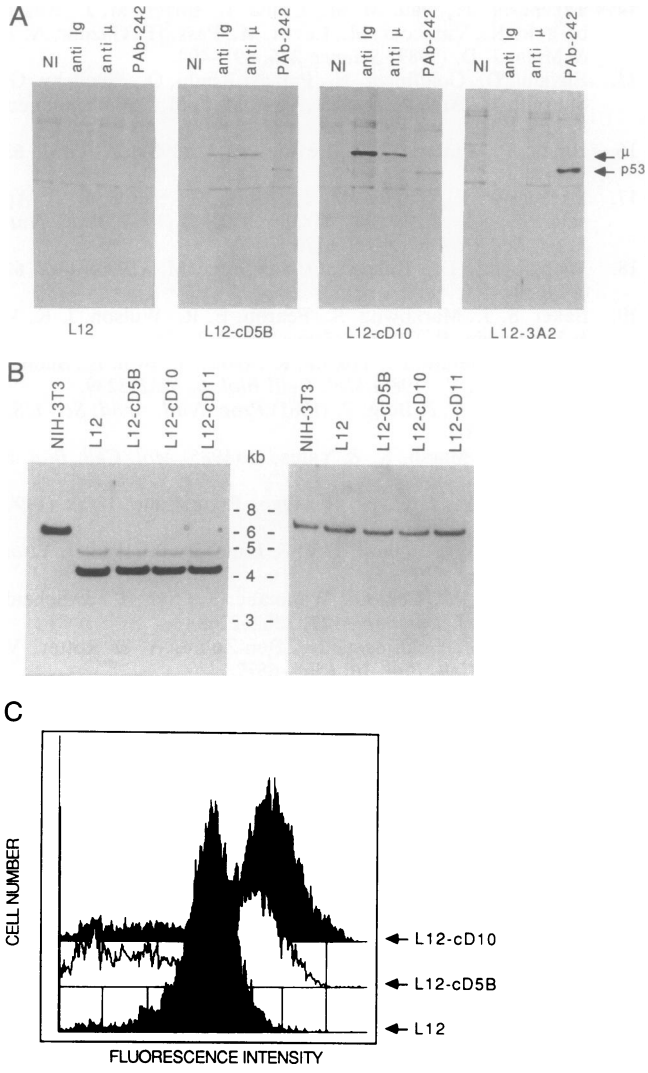

FIG. 4. Effect of wild-type p53 protein expression on differentiation of L12 cells. (*A*) Expression of immunoglobulin μ heavy chain in L12 cells. Cells were lysed and immunoprecipitated with nonimmune serum (NI), goat anti-mouse total immunoglobulin (Ig) antibodies, goat anti-mouse μ antibodies, and anti-p53 monoclonal antibody PAb-242. (*B*) Southern blot analysis of structure of μ heavy-chain and κ light-chain genes. High molecular weight DNA was digested with *Eco*RI and probed with μ heavy-chain-specific probe (*Left*) or with *Eco*RI and *Bam*HI and probed with κ light-chain-specific probe (*Right*). (*C*) B220 expression in L12-derived cell lines. Exponentially growing intact cells were incubated with rat anti-mouse B220 antibodies and analyzed by flow cytometry. The logarithmic distributions of fluorescein-derived fluorescence intensity of the various cell lines are presented.

any of the L12-cD cell lines examined. Furthermore, when the immunoglobulin κ light-chain locus was analyzed, no rearrangements were found in L12 or L12-cD-derived clones (Fig. 4*B*).

The B220 antigen is a B-cell surface marker that is expressed at relatively low densities on bone marrow pre-B cells and in greater amounts on mature B-lineage cells (25, 31). A comparison between the parental L12 cells and the L12-derived clones expressing the wild-type p53 indicated that the latter expressed higher levels of the B220 cell surface marker. Flow cytometry of stained cells showed significantly higher fluorescence densities of B220 on the cell surface of all L12-cD cell lines compared with the parental L12 cells (Fig. 4*C*). This suggests that L12-cD-derived clones represent a more advanced stage along the B-cell differentiation pathway.

DISCUSSION

To characterize the physiological function of wild-type p53, we studied the expression of the wild-type gene in a cell system free of any endogenously expressed p53 protein. Studying L12 cells offered two main advantages. (*i*) Because these cells lack the expression of any endogenous p53 protein, introduction of wild-type p53 was expected to represent the authentic unadulterated function of the p53 protein. (*ii*) The fact that L12 cells are pre-B cells that can differentiate under *in vitro* conditions permitted us to study the involvement of wild-type p53 in cell differentiation.

The conclusion that wild-type p53 triggered cell differentiation was based on the observations that expression of this protein induced the synthesis of immunoglobulin μ chain in the L12 nonproducer cells and that cells expressing wild-type p53 exhibited a higher level of the B220 cell surface marker. It is not clear whether wild-type p53 directly induced the expression of these two genes or whether one of them or yet a third gene was initially transactivated by wild-type p53, leading in turn to further cell differentiation.

Both immunoglobulin alleles of L12 cells are rearranged and expression of wild-type p53 did not induce any further changes in their structure. This suggests that wild-type p53 was involved in a process that eventually led to the transactivation of these already rearranged genes. Activation of the μ heavy-chain gene expression can be mediated by a large number of cellular proteins. These cellular proteins function through their direct binding to specific motifs found in the heavy-chain enhancer sequences (32–34). The possibility that wild-type p53 functions in a similar way is strongly supported by the fact that this DNA-binding protein (35, 36), which is actively transported into the nucleus (26, 37, 38), was shown to function as a transcription factor (39, 40). Wild-type p53 may be involved in the network of genes that eventually induce transactivation of the μ heavy-chain promoter (32–34).

Pre-B cells represent an early developing cell population capable of undergoing self-renewal involving proliferation and, at the same time, capable of entering a differentiation pathway leading to cell maturation that has not been found to be coupled with terminal cell growth arrest (29, 30). We propose that the wild-type p53 expressed in these pre-B cells pushes them to advance to a more mature stage; they then have a lower renewal capacity than the parental cells but are on an accelerated differentiation pathway. The net balance of these cells still represents a growing population, which nevertheless exhibits a profile of more differentiated cells. L12 pre-B cells were induced to differentiate only when expressing wild-type p53, but not when expressing mutant p53 or drug-resistance genes. This strongly suggests that differentiation of these pre-B cells is a specific result of wild-type p53 activity rather than clonal selection that might have occurred upon gene transfer and drug selection.

The apparent disagreement between our present study, where we report on the establishment of stably growing clones expressing wild-type p53, and previous reports, showing unequivocally that overexpression of the wild-type p53 leads to terminal growth arrest, can be explained because these experimental models are concerned with the expression of wild-type p53 in different microcellular environments. It could be that in transformed glioblastoma cells (17) or fibroblasts (18) that are probably more advanced in their differentiation pathways expression of wild-type p53 induced terminal differentiation, leading to a net effect of growth arrest. It can therefore be assumed that p53 induces cell differentiation at either an earlier or a later phase on the route of cell differentiation. Manifestation of growth arrest induced by wild-type p53 is most likely in inverse correlation with the cell differentiation state. When introduced at an early phase

of cell differentiation, wild-type p53 causes almost no growth arrest, whereas at a more advanced phase, cell growth arrest is predominant. It should be mentioned, however, that differences in the effect of wild-type p53 expression in the various experimental systems used could be due to variations in the subcellular levels of protein expressed by the different expression vectors used in the different cell lines studied. Also, various cell lines may tolerate different threshold levels of p53 before exhibiting total growth arrest.

In conclusion, we suggest that wild-type p53 functions as a physiological cell differentiation factor. Introduction of wild-type p53 into cells at an early phase of their differentiation pathway induces them to advance to a more differentiated stage. When cells are already at a more advanced phase of their differentiation, expression of wild-type p53 pushes them to terminal differentiation that is accompanied by growth arrest. However, the possibility that these effects of induction of cell differentiation are specific for pre-B cells only cannot be excluded.

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