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p53 Functions as a Cell Cycle Control Protein in Osteosarcomas

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Mutations in the p53 gene have been associated with a wide range of human tumors, including osteosarcomas. Although it has been shown that wild-type p53 can block the ability of E1a and *ras* to cotransform primary rodent cells, it is poorly understood why inactivation of the p53 gene is important for tumor formation. We show that overexpression of the gene encoding wild-type p53 blocks the growth of osteosarcoma cells. The growth arrest was determined to be due to an inability of the transfected cells to progress into S phase. This suggests that the role of the p53 gene as an antioncogene may be in controlling the cell cycle in a fashion analogous to the check-point control genes in *Saccharomyces cerevisiae*.

p53 is a nuclear phosphoprotein that has recently been found to have many of the properties of a tumor suppressor (17, 20). It was first detected by immunologic methods in simian virus 40 (SV40)-transformed cells (12, 37, 39). The gene encoding p53 was initially considered a dominant growth-activating oncogene because experiments that used what was assumed to be the wild-type p53 gene indicated that it could cooperate with a *ras* oncogene in the transformation of rat embryo fibroblasts (18, 35, 44). More recent analyses indicate that the p53 gene used in these transfection experiments was in fact mutant and that the wild-type p53 gene is unable to transform rat embryo fibroblasts in cooperation with *ras* (16, 31). In addition, many p53 gene mutations that affect various portions of p53 all cooperate with *ras* to transform cells (16, 17, 20, 31). It has also been suggested that mutant p53 acts in a dominant fashion, with the mutant protein having an ability to be dominant over the wild type. The proposed mechanism for this involves formation of oligomers (36). This ability to oligomerize, combined with the fact that mutant p53 frequently forms complexes with the heat shock proteins, in particular heat shock complex 70 (hsc70) (6, 21, 53, 54), means that p53 may become trapped in a complex with hsc70. This model both predicts that wild-type p53 can become functionally inactivated through the presence of mutant p53 and explains the ability of exogenous mutant p53 to transform cells despite the presence of endogenous wild-type p53.

The role of p53 in human tumors has also undergone a recent reappraisal. In initial surveys of over 100 tumors examined for p53 alterations, osteosarcomas were the only tumors with detectable rearrangements in this gene (40). Assignment of the p53 gene to the short arm of chromosome 17 (34), where several common human tumors have frequent loss of alleles, led to more careful studies of p53 in those tumors. It was found that all colon cancers with single allelic deletions had p53 gene mutations in the remaining allele (2, 43). Similar studies now indicate that lung cancers (38, 43, 56), brain tumors and breast carcinoma (43), hepatocellular carcinoma cell lines (4), and chronic myelogenous leukemia cells in blast crisis (1) also undergo p53 gene alterations. The wide spectrum of tumors with p53 changes suggests that p53 may exert a fundamental effect in controlling transformation.

Osteosarcomas are rare bone tumors that occur primarily in adolescents and young adults. There are several indications that inactivation of the retinoblastoma (*Rb*) gene is important for osteosarcoma formation. These tumors frequently have detectable rearrangements in the *Rb* gene and loss of the *Rb* gene product p105 (23), and patients with hereditary retinoblastoma are at greatly increased risk for osteosarcomas (13). Reintroduction of the retinoblastoma gene into osteosarcomas that lack functional retinoblastoma protein severely limits the growth of these tumors (29). Osteosarcomas are also known to undergo numerous other genetic alterations. A frequent karyotypic change is deletion of the short arm of chromosome 17, where the p53 gene has been found to be localized (34). As noted above, several reports have suggested that osteosarcomas have changes in gene p53 (15, 40, 46).

This article considers the importance of the p53 gene control pathway in established osteosarcoma cell lines. Our data indicate dramatic differences in the effects of mutant and wild-type p53 genes on osteosarcoma cell growth. The use of flow cytometry on transiently transfected cells is developed as a novel technique to determine where in the cell cycle wild-type p53 gene functions to control growth.

MATERIALS AND METHODS

Cell culture and DNA transfection. The following nine cell lines were obtained from the American Type Culture Collection: Saos-2, HTB 85; MG-63, CRL 1427; G-292, CRL 1543; KHOS-240S, CRL 1545; KHOS-312S, CRL 1546; 143B, CRL 8303; and U-2OS, HTB 96. The 8842 and 7922 cell lines were kind gifts from James Epstein at the Massachusetts Eye and Ear Infirmary (14). The OHS-50T and HTLA-161 cell lines were generously provided by Emil Bogenmann at the Los Angeles Children's Hospital (19). All cell lines were maintained in Dulbecco modified Eagle

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medium (DME) with glucose (4.5 g/liter), L-glutamine, and sodium pyruvate. The medium was supplemented with 10% heat-inactivated fetal calf serum and antibiotics. Cells were grown at 37°C in a humidified 5% CO_2 atmosphere.

Cells were transfected at 50 to 70% confluence by the calcium phosphate procedure (48). Each 10-cm plate with 1 × 10^6 to 5 × 10^6 cells received 1.25 ml of a calcium phosphate (pH 7.05) precipitate containing 20 μg of plasmid DNA for stable transfections and 60 to 80 μg for the transient transfections. Increased amounts of DNA were used in the transient transfections in order to increase the levels of p53 expressed and to increase the proportion of cells that were able to be transfected. The precipitate was left on the cells for 4 h before a 2-min incubation with 15% glycerol, followed by three phosphate-buffered saline (PBS) washes. Transient assays were performed 48 to 60 h after the glycerol shock. Stable cell lines were generated by transfection with a neomycin resistance gene-containing plasmid or by cotransfection with pSVXR1 and pSV2NEO at a 1:10 ratio. After 2 days, cells were split 1:6 into medium containing 500 μg of Geneticin (G418; GIBCO) per ml. Single-cell clones were picked after 4 to 6 weeks.

Southern blots. Southern blotting techniques and hybridizations were performed as described previously (50) with the capillary transfer method, Hybond nylon membranes (Amersham), and non-formamide-containing hybridization solutions. Hybridization probes were made from gel-isolated DNA fragments that were labeled by random priming with the Klenow fragment of DNA polymerase and [α-^{32}P]dCTP (19).

Immunoprecipitations. Cell lines were metabolically labeled with 50 μg of methionine translabel (ICN) per ml in 2.5 ml of methionine free DME plus 2% dialyzed fetal calf serum for 2 h. Cells were rinsed in ice-cold PBS twice before lysis in 1 ml of ELB (250 mM NaCl, 0.1% Nonidet P-40, 50 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid, pH 7.0], 1 mM phenylmethylsulfonyl fluoride [Sigma], 5 mM EDTA, 0.5 mM dithiothreitol). The lysate was scraped from the plate, centrifuged, and stored at −80°C. Lysates were preadsorbed with fixed staphylococcal protein A overnight at 4°C and then incubated with polyclonal antibody PAb122 before precipitation and loading on 8% acrylamide gels.

Radioimmunoassays. Radioimmunoassays were carried out in 96-well filter-bottomed plates (V&P Scientific Inc., San Diego, Calif.). Briefly, cell homogenates were prepared from cells in PBS–0.1% sodium azide with a Polytron homogenizer. Homogenates containing four different concentrations of protein were applied directly to the filter papers in triplicate. After adsorption of proteins onto the filters by incubation for 30 min, excess binding sites were saturated by incubation with calf serum. Then, 100 μl of ^{125}I-labeled PAb122 antibody solution (100,000 cpm in calf serum containing 1% nonspecific mouse immunoglobulin G [IgG] ascites fluid) was added and incubated for 16 h at 4°C. Filters were then washed three times with 20% calf serum in PBS and counted with a gamma counter.

Plasmids. pC53SN3 is an expression construct that contains a p53 cDNA driven by a cytomegalovirus (CMV) promoter and the neomycin resistance gene driven by an SV40 promoter-enhancer. The expression vector was derived from plasmid BCMGNeo-IL2 after excision of the human beta-globin sequence and bovine papillomavirus sequences with *Bam*HI and *Not*I. Next the *Xho*I site was changed to a *Bam*HI site by linker addition. The entire coding sequence of p53 cDNA (an 1,800-bp fragment extending from an *Xba*I site 130 bp 5′ of the ATG to another *Xba*I site 492 bp 3′ of the stop codon) was cloned into the *Bam*HI site. pC53SCX3 was constructed by the same methods. The pSVXR1 construct contains a 1,600-bp p53 cDNA fragment extending from the *Xba*I site 5′ to the ATG to an *Eco*RI site 3′ of the stop codon. This was cloned into the expression vector pKC4, which contains an SV40 promotor.

Immunofluorescence. Cells were transfected 24 h before being plated onto sterile multiwell glass slides (Polysciences Inc.) at a density of about 50% on the night before staining in Dulbecco modified Eagle medium with 15% fetal calf serum and antibiotics. Nontransfected cells were treated similarly. The cells were rinsed with serum-free medium and with nonsterile PBS, then fixed with 70% ethanol for 10 min, and permeabilized with 0.1% Triton X-100 in PBS for 10 min. All steps were carried out at room temperature. Cells were blocked with 1% bovine serum albumin (BSA; Sigma) in PBS for 30 min. Primary antibodies were used at concentrations of either 1:500 or 1:1,000 in 1% BSA–PBS, incubated for approximately 1 h, and then removed with several PBS rinses.

The p53 antibody PAb122 was obtained from a hybridoma cell line (American Type Culture Collection). The staining antibody goat anti-mouse IgG (Caltag) was applied at a concentration of 1:100 for 45 min and then removed with PBS. The slides and cover slips were mounted in an 80% glycerol solution with *p*-phenylenediamine (Sigma) at 1 mg/ml. Epifluorescence microscopy was done with a Zeiss fluorescence microscope. A Nikon UFX-IIA camera system and Kodak TriX ASA 400 and Tmax ASA black and white 35-mm films were used to photograph the specimens.

PCR amplification and sequencing. p53 cDNA which had been stably transfected into the Saos-2 cell line was amplified from total genomic DNA by the polymerase chain reaction (PCR) with two primers spaced 1,270 nucleotides apart and spanning the entire coding region. The sense primer contained an *Eco*RI site at its 5′ end, and the antisense primer contained a *Bgl*II site at its 3′ end. The DNA template and primers were added to a PCR buffer containing 50 mM KCl, 10 mM Tris hydrochloride, and 1.5 mM $MgCl_2$, to which dNTPs (200 μM each) and 2.5 U of *Thermus aquaticus* polymerase (Cetus) were added. The reaction was amplified for 40 cycles in a Perkin-Elmer Thermocycler (40 s of denaturation at 94°C, 2 min of annealing at 55°C, 2 min of elongation at 72°C). A 7-min elongation step was added after the last cycle to complete any partial extensions. The PCR product was digested with *Eco*RI and *Bgl*II and ligated into a vector, pBSK (Stratagene), for sequencing. Three single colonies from two separate PCR reactions were sequenced in their entirety on both strands by the dideoxy chain termination technique with a T7 polymerase kit (Pharmacia). The U-2OS genomic DNA was amplified between exons 5 and 9 and sequenced as previously described (43). The sequencing reaction mixes were separated by electropheresis on gradient 6% polyacrylamide gels.

Flow cytometry and cell cycle analysis. Exponentially proliferating Saos-2 cells were transfected with either the wild-type p53 gene expression construct pC53SN3 or the mutant p53 gene construct pC53CX3. Twenty-four hours later, the cells were incubated for 24 h in either nocodazole (40 ng/ml; Sigma) or 0.1 mM hydroxyurea (Sigma). Then, 4 × 10^6 cells were rinsed with PBS, fixed in 95% ethanol for 15 min, rinsed again, suspended in 1% BSA–PBS containing 200 U of RNase (Sigma R5000) per ml, and agitated for 30 min at 37°C. The cells were then divided into two portions, for

control or for p53 staining, in which case they were suspended in 0.25 ml of PAb122 (1:1,000 in 1% BSA–PBS). The cells were agitated for 1 h at 22°C, rinsed, suspended in 0.25 ml of fluorescein isothiocyanate (FITC)-conjugated goat $F(ab')_2$ mouse IgG (1:1,000 in BSA-PBS) (Caltag n35001) for 1 h, rinsed again, and counterstained by suspension in 0.5 ml of PBS with 10 μg of propidium iodide (Calbiochem) per ml. Flow cytometry was performed on a custom-built Cytomult flow cytometer equipped with a 15-mW 488-nm argon laser. DNA content measurements from propidium iodide were collected with a photomultiplier masked with a 630-nm long-pass filter, and FITC-p53 fluorescence was collected through a 520-nm band pass filter. Fluorescent signals were processed into histograms and scattergrams and gated with the 4Cyto software system (Shapiro Flow Cytometer Lab, Cambridge, Mass.).

RESULTS

p53 alterations in osteosarcoma cell lines. To begin our studies, we examined p53 DNA sequences in several osteosarcoma cell lines by Southern blot analysis. Cell lines that contained the intact p53 gene exhibited two *Hin*dIII fragments of 7.0 and 2.5 kb when they were probed with a cDNA clone that encoded exons 2 through 11 of the p53 gene. As can be seen in Fig. 1A, the Saos-2 and 8842 cell lines had deletions that included all the sequences detected by the probe, and the HTLA-161 cells contained a p53 gene rearrangement which was confirmed by other restriction digests (data not shown). Thus, gross p53 gene alterations were detectable in three of the independently cloned osteosarcoma cell lines.

Loss of suppressor gene function can be achieved by more subtle alterations, such as point mutations or small deletions that result in no protein or a mutant protein. We therefore analyzed the osteosarcoma cell lines by immunoprecipitation of the cell lysates with a p53 antibody, PAb421. As is shown in Fig. 1B, three additional cell lines, MG-63, G-292, and 7922, that had no alterations by the Southern blot analysis did not express detectable levels of p53 protein. These cell lines also had no detectable p53 protein by Western immunoblot analysis (data not shown).

It has been shown previously that whereas wild-type p53 does not bind detectable amounts of hsc70, many mutant p53s bind the heat shock complex 70 proteins (hsc70s) (21). Therefore, to screen for subtle p53 mutations, immunoprecipitations of [^{35}S]methionine-labeled lysates were also examined for whether the p53 bound hsc70. hsc70 apparently coprecipitated with p53 in all HOS-derived cell lines (bracketed in Fig. 1B) as well as in OHS50T and 143B, strongly suggesting that these cell lines contained mutant p53. When Western blots that were immunoprecipitated with a p53 antibody were probed with an anti-hsc70 monoclonal antibody, we confirmed these bands as hsc70 (data not shown). The specific p53 mutation in the HOS cell line and related sublines has recently been described (46). Because there are differences in tumorigenicity between HOS sublines (7), we analyzed these sublines separately. No differences in p53 protein levels or hsc70 binding were found in tumorigenic (KHOS-NP) versus nontumorigenic (KHOS-312H and KHOS-240S) sublines.

Only one osteosarcoma cell line, U-2OS, was found to express a p53 that did not bind hsc70. Both alleles of chromosome 17p were noted to be present (data not shown) by probing *Hin*fI-digested U-2OS genomic DNA as described previously (43). The level of p53 in the U-2OS cells

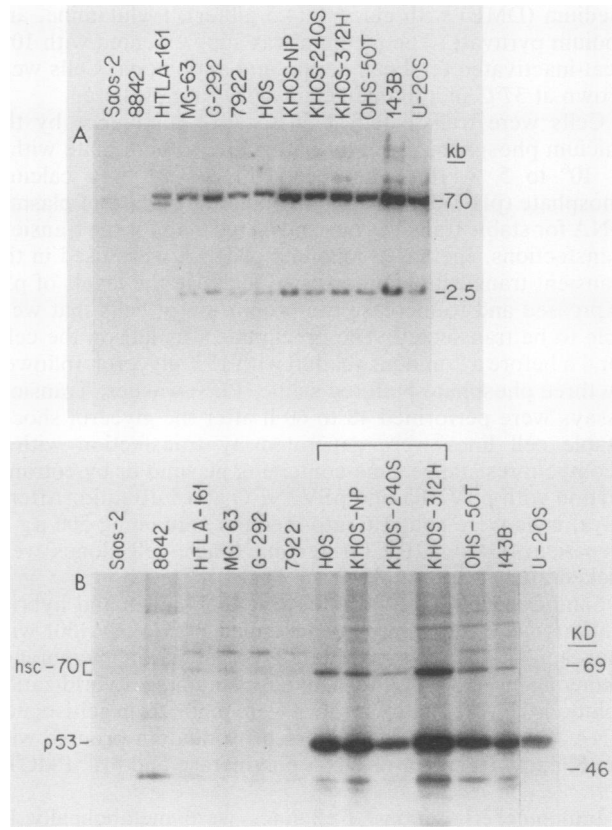

FIG. 1. Analysis of gene p53 alterations in osteosarcoma cell lines. (A) Southern blot analysis. Ten-microgram samples of *Hin*dIII-digested genomic DNA from osteosarcoma cell lines were subjected to Southern blot analysis. The two genomic p53 fragments, 7.0 and 2.5 kb in length, expected from *Hin*dIII digestion of intact genomic DNA from the intact p53 gene are indicated. (B) Immunoprecipitates from [^{35}S]methionine-labeled osteosarcoma cells were run on an 8% polyacrylamide gel. p53 was immunoprecipitated with the monoclonal antibody PAb421. The hsc70 proteins and p53 are identified. Four of the cell lines are bracketed because they were derived from the same original osteosarcoma. KD, Kilodaltons.

was determined by radioimmunoassay to be at the same level as in normal diploid fibroblasts (data not shown). The gene encoding p53 from U-2OS cells was therefore sequenced from exons 5 through 10, where all previous p53 mutations have been located (2, 43, 56), and no mutations were found. The observations that both p53 gene alleles were present, that the p53 levels by radioimmunoassay were comparable to the level in normal diploid fibroblasts, and that mutations in the susceptible p53 coding sequences were lacking all suggest that U-2OS cells contain apparently wild-type gene p53. In summary, 9 of the 10 osteosarcoma cell lines were shown to have altered p53 genes or reduced expression of the p53 gene product.

Effect of p53 gene on the growth of osteosarcoma cell lines. Wild-type and mutant p53 gene expression vectors that function in osteosarcoma cell lines were constructed. Figure 2 shows a map of the wild-type p53 gene expression construct pC53SN3. Wild-type p53 cDNA was cloned from normal human polyadenylated RNA and ligated into the eucaryotic expression vector pCMV-NEO-Bam. Two mutant p53 gene expression constructs were used as controls. pC53CX3 is identical to the wild type except that it contains

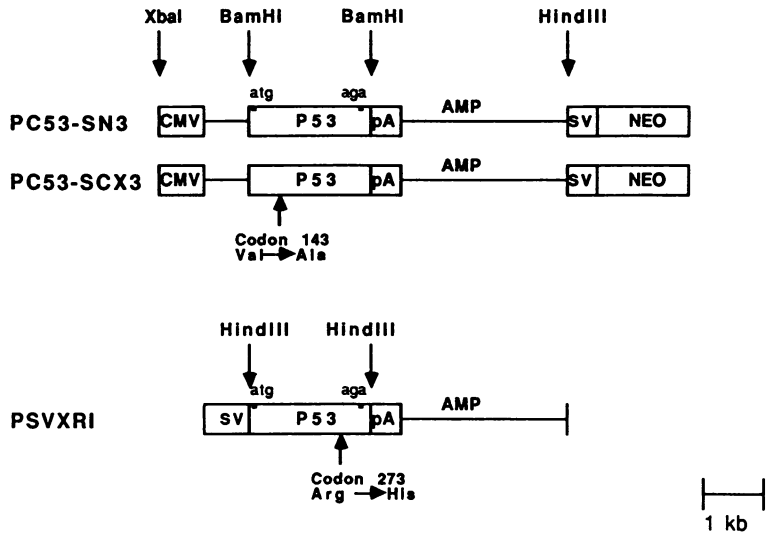

FIG. 2. Wild-type and mutant p53 cDNA expression constructs. Schematic diagrams of p53 expression vectors. Plasmids were constructed as described in the text. The wild-type p53 cDNA construct pC53SN3 and the mutant p53 cDNA construct pC53SCX3 differ by a single nucleotide that results in an alanine instead of a valine at codon 143 in p53. Digestion with *Bam*HI releases an 1,800-bp cDNA fragment that encodes p53. The other mutant gene p53 expression construct, pSVXR1, has a single mutation that results in histidine replacing an arginine at codon 273 in p53. Digestion with *Hin*dIII releases a 1,600-bp cDNA fragment that encodes p53. pA, Polyadenylation signal; AMP, ampicillin resistance gene; SV, SV40 promoter/enhancer; CMV, CMV promoter/enhancer; NEO, neomycin resistance gene. The positions of the initiation and termination signals for p53 translation are also included.

a p53 cDNA clone from a colon carcinoma xenograft, CX3, which has a point mutation resulting in a valine to alanine change at codon 143 (2). The second mutant construct, pSVXR1 (provided by E. Harlow), contains the cDNA from the epidermoid carcinoma cell line A431. This p53 gene encodes an arginine to histidine change at codon 273. The pSVXR1 mutant p53 construct contains no selectable marker; therefore, it was cotransfected with pSV2NEO.

To test whether these vectors could drive the synthesis of p53 protein, we transiently transfected Saos-2 cells with each of the three p53 expression constructs by the standard calcium phosphate technique. All three expression constructs synthesized p53 which was easily detectable by immunoprecipitation 24 h after transfection (data not shown). Untransfected Saos-2 cells contained no detectable p53 protein, whereas transfected cells synthesized protein (data not shown).

In order to assess the function of wild-type p53, we introduced wild-type and mutant p53 genes into three different osteosarcoma cell lines. Transfection of wild-type p53 gene into cells containing large amounts of mutant p53 might produce a different effect than transfection into a cell line lacking p53. Additionally, if the effect of mutant p53 requires hsc70 binding, transfection of the wild-type p53 into a cell line with endogenous p53 that does not bind hsc70 might provide a different result. We therefore decided to reintroduce p53 into Saos-2 cells, which have no p53; into U-2OS cells, which have apparently wild-type p53 that does not bind hsc70; and into KHOS-240S cells, which contain large amounts of mutant p53 stabilized by hsc70.

The frequency at which stable clones arose in G418 selection after transfection is listed in Table 1 as colonies per microgram of transfected plasmid. Transfection of the wild-type p53 construct, pC53SN3, resulted in approximately 100-fold fewer colonies than transfection with the mutant p53 constructs. This reduction in colony number was seen in every experiment with each of the osteosarcoma cell lines. To exclude the possibility that the decrease in the number of colonies reflected differences in transfection efficiencies, cells were transiently assayed for the frequency of p53 expression by immunofluorescent techniques. Transfection of 20 μg of pC53SN3, pC53SCX3, and pSVXR1 per 10^6 Saos-2 cells resulted in 7, 13, and 11% of the cells expressing p53, respectively. Therefore, the significantly fewer colonies suggested that wild-type p53 but not mutant p53 had a growth-inhibitory effect.

We then examined p53 cDNA integration in the colonies recovered from the transfections. Genomic DNA from the lines was analyzed by Southern blot for intact integration of exogenous p53. The analysis of some representative cell lines is shown in Fig. 3A. The endogenous p53 gene was detected in the first six lanes as a 7.8-kb fragment when the genomic DNA was digested with *Bam*HI or as 7.0-kb and 2.5-kb fragments in lanes 7 to 11 when the genomic DNA was digested with *Hin*dIII. The integrated exogenous p53 cDNA fragment should be 1.8 kb in length from the pC53SCX3 and pC53SN3 constructs when digested with *Bam*HI, as in lanes 1 to 6, and it should be 1.6 kb in length when the integrated pSVXR1 vector is digested with *Hin*dIII, as in lanes 7 to 11. Whereas three of the four pC53SCX3 and four of the five pSVXR1 colonies that are shown had proper integration of the exogenous mutant p53 cDNA, neither of the two colonies that were transfected with the wild-type p53 construct pC53SN3 shown here had intact integration of p53 cDNA.

Table 1 summarizes our ability to detect intact integration of p53 cDNA in the single-cell clones transfected with the three expression constructs. Only 5 of the 76 rare clones that grew after transfection with the wild-type p53 construct contained intact integration of the p53 cDNA, even though the presence of the neomycin resistance gene was detected in all 76 colonies by reprobing with a neomycin resistance gene cDNA fragment (data not shown). This is in contrast to transfection with the two mutant p53 constructs, where intact integration of p53 could be detected at high frequency in all three osteosarcoma cell lines tested. We conclude that

TABLE 1. Detection of p53 reintroduction into osterosarcoma cell lines

Cell line	Exp	No. of G418 colonies/μg of DNA (pC535N3 + pSVXR1)	No. positive/no. tested (% positive) pC53SN3		pSVXR1		No. of G418 colonies/μg of DNA (pC53CX3)	No. positive/no. tested (% positive) pC53CX3	
			Integration of p53 cDNA	Expression of p53	Integration of p53 cDNA	Expression of p53		Integration of p53 cDNA	Expression of p53
Saos-2	T512	3	1/1	0/1[a]	6/6	6/6	227		
	T718		1/3	0/1				6/6	3/3
	T726		0/5	0/1					
	T81			0/2				0/2	3/5
	T88							3/3	
	T926								4/4
U-2OS	T512	4	0/15	—[b]	1/1	—	320		
	T63		1/14	—		—		12/13	4/4
	T88		0/14	—		—		8/8	6/6
KHOS-24OS	T512	2	0/9	—	4/6	—	90		—
	T63		0/10	—		—			—
	T88		2/5	—		—		—	—
All lines			5/76 (7)	0/5 (0)	11/13 (85)	6/6 (100)		29/33 (88)	20/22 (91)

[a] Full-length protein underwent mutation.
[b] —, Exogenous p53 expression could not be assessed.

there is a high selection pressure against the cell lines with proper integration of the wild-type p53.

We next examined whether p53 protein was expressed in the transfectants. The stable cell lines were screened by immunoprecipitation of ^{35}S-labeled cell lysates with p53 antibodies. The analysis of several cell lines is shown in Fig. 3B, and the analysis of all the cell lines is summarized in Table 1. One of the two Saos-2 cell lines that had intact integration of the wild-type p53 cDNA synthesized p53 protein of the correct size; the other synthesized no wild-type p53 (Fig. 3B, lanes 3 and 4). Unlike wild-type p53, the p53 protein synthesized in this cell line, Saos-2 T512SN3.1, bound detectable amounts of hsc70, and therefore it probably underwent some mutation.

PCR was used to amplify the p53 cDNA fragment, which was then sequenced. Three clones from two separate PCRs were sequenced in their entirety in both directions. All three clones had alterations at codons 266 and 306 (Fig. 4). This means that none of the rare Saos-2 colonies transfected with the wild-type p53 cDNA were able to tolerate its presence. In contrast, 16 of the 18 numerous Saos-2 colonies transfected with the two mutant p53 constructs were able to direct the synthesis of the mutant p53 protein (Fig. 3B, lanes 1, 2, and 5 to 7). Essentially the same results were found when the gene encoding p53 was transfected into two other osteosarcoma cell lines. Figure 3B (lanes 11 and 12) shows that it was possible to detect the synthesis of mutant p53 directed by the pC53SCX3 construct in the U-2OS cells because unlike the endogenous p53 gene, the newly introduced mutant p53 gene bound hsc70. All 10 U-2OS cell lines transfected with pC53SCX3 expressed mutant p53 that bound hsc70. In U-2OS and KHOS-240S, there was no significant increase in the level of p53 protein whether they were transfected with the wild-type or mutant expression vectors. The level of p53 protein in the stable transfectants was therefore presumably not in great excess over that produced endogenously. Cell lines that for this reason could not be analyzed for exogenous p53 protein are indicated in Table 1. In summary, transfection of wild-type p53 gene significantly reduced the number of stable colonies that could be formed in the three osteosarcoma cell lines. The colonies that were able to form infrequently had intact integration of the p53 cDNA. The rare clones with apparently intact exogenous wild-type p53 DNA did not direct the synthesis of wild-type p53 protein.

Sequences controlling subcellular localization. The sequence data obtained in our analysis of the Saos-T512SN.1 transfectant revealed an interesting mutation, which led us to investigate the subcellular localization of p53 in this mutant cell line. The G to A change 917 nucleotides from the ATG resulted in replacement of the arginine by glutamine at codon 306, very close to the putative core nuclear localization signal at positions 317 to 322 (10) and a major phosphorylation site at codon 315 (3). Immunofluorescence was used to determine whether this loss of a positive charge near the core nuclear localization signal altered the subcellular localization of p53. Figure 5 shows that p53 was not detectable in untransfected Saos-2 cells and that it was found in the nucleus of KHOS-240S cells, which contain mutant p53. Saos-2 cells, whether stably transfected with pC53SCX3 or transiently transfected with pC53SN3 and pSVXR1, all made p53 that was localized in the nucleus. In contrast, p53 was not localized to the nucleus of the Saos-2 T512SN3.1 cell line. Although the mutant codon 306 is not within the published core nuclear localization sequence, its proximity to it may indicate that this signal was perturbed by a nearby mutation, especially one which results in a change in the positive charge. Disruption of subcellular localization represents a novel potential mechanism for the functional inactivation of p53, as all other mutant p53s we have studied have been localized in the nucleus (data not shown).

How does wild-type p53 suppress the growth of osteosarcoma cells? The data shown above demonstrate that overexpression of wild-type p53 is not tolerated in osteosarcoma cells. Since p53 is a nuclear phosphoprotein that undergoes cell cycle-related changes in quantity and phosphorylation (41, 42, 45, 49), it seemed possible that p53 blocked osteosarcoma cell proliferation by deregulating the normal progression through the cell cycle. To test this hypothesis, we designed an experiment with immunofluorescence and flow cytometry. Transient transfection of p53 cDNA constructs into cells that lack p53 (Saos-2 cells) and use of the anti-p53 antibody PAb122 permitted cells to be analyzed for reintroduction of p53. Fluctuations in the p53 content during the cell cycle were analyzed by variations in p53 immunofluo-

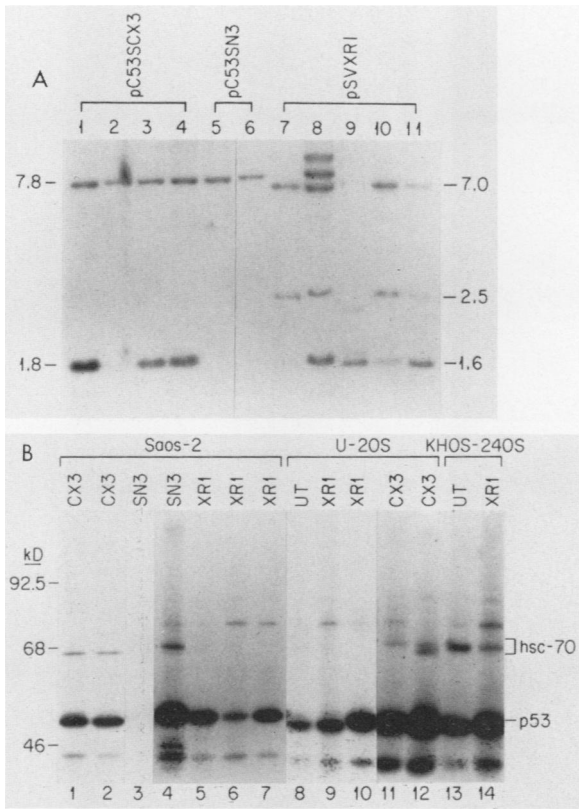

FIG. 3. Analysis of p53 in osteosarcoma cell transfectants. (A) Evidence for intact integration of exogenous p53 cDNA. Southern blot analysis of single-cell clones transfected with p53 expression constructs. Ten-microgram samples of genomic DNA were digested with *Bam*HI (lanes 1 to 6) or *Hin*dIII (lanes 7 to 11) and probed with a p53 cDNA fragment. Lanes 1 to 4, DNA from cells transfected with pC53SCX3; lanes 5 and 6, DNA from cells transfected with pC53SN3; lanes 7 to 11, DNA from cells transfected with pSVXR1. Lanes 1 to 5 and 12 contain DNA from transfected U-2OS cells; lanes 6 and 8 to 10 contain DNA from transfected Saos-2 cells; lanes 7 and 11 contain DNA from transfected KHOS-240S cells. (B) Detection of p53 expression. Lysates from [^{35}S]methionine-labeled osteosarcoma cells were immunoprecipitated with the monoclonal antibody PAb421. Lysates are shown from Saos-2 cells in lanes 1 to 7. Lanes contain lysates from cell lines transfected with pC53SCX3, (T512CX3.6 [lane 1] and 3.10 [lane 2]), pC53SN3 (T718SN.3 [lane 3] and T512SN3.1 [lane 4]), and pSVXR1 (T512XR1.3 [lane 5], 1.13 [lane 6], and 1.4 [lane 7]). Lysates from U-2OS cells are in lanes 8 to 12. Lanes contain lysates from untransfected (UT) cells (lane 8) or cells transfected with pSVXR1 (T512XR1.1 [lane 9] and 1.2 [lane 10] or pC53SCX3 (T63CX3.6 [lane 11] and 3.11 [lane 12]). Lysates from untransfected KHOS-240S are in lane 13 and transfected with pSVXR1 in lane 4. Molecular mass standards are indicated in kilodaltons (kD). p53 and the hsc70 proteins are identified.

rescence as detected by flow cytometry within various portions of the cell cycle. Only a two- to fourfold variation in p53 was noted (data not shown), confirming previous reports that it was possible to detect similar levels of p53 throughout the cell cycle (9).

The distribution of cells within the cell cycle can be determined by their DNA content through measurement of fluorescence emission after propidium iodide staining. There is a doubling of the DNA content in the progression from the G_1 phase to the G_2/M phase, when DNA synthesis occurs during S phase. Cells in the G_2/M phase maintain this doubled DNA content until the cells divide and reenter the

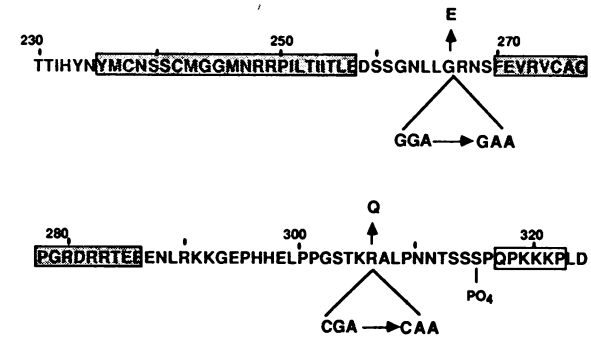

FIG. 4. Identification of p53 mutations in the Saos-2 T512SN.1 transfectant cell line. p53 amino acid sequence between codons 230 and 324. Shaded boxes designate sequences in highly conserved domains. An unshaded box identifies residues in the core nuclear localization signal (10). A phosphate group is attached to one of the putative phosphorylation sites at serine 315 (PO_4). Both the nucleotide alterations and the resultant amino acid changes at codons 266 and 306 are presented.

G_1 phase. Staining with propidium iodide thereby permits determination of cell cycle distribution as the second parameter. By use of flow cytometry, cells can be analyzed for both p53 expression and distribution within the cell cycle.

In order to determine whether p53 altered cell cycle progression, Saos-2 cells were first transfected with the gene encoding p53. To test the level of p53 protein that was transiently expressed in these cells, radioimmunoassays with ^{125}I-labeled PAb122 were performed. Saos-2 cells, normal fibroblasts, and the KHOS-240S cells had 29 ± 13, 66 ± 5, and 425 ± 51 cpm of bound antibody per 10 μg of protein, respectively (mean ± SD). Saos-2 cells transiently transfected with 60 to 80 μg of wild-type pC53SN3 or mutant pC53SCX3 DNA per 2×10^6 cells had 17,645 ± 1,932 and 12,368 ± 948 cpm of bound antibody per 10 μg of protein, respectively. Transfection of large amounts of these expression constructs allowed transient expression of p53 at levels greater than 30-fold higher than that found in the KHOS-240S cells and several-hundred-fold higher than in normal fibroblasts.

In order to determine whether p53 altered cell cycle progression, transfected cells were treated with drugs used to synchronize cells at specific cell cycle stages (52) and then analyzed for DNA content. Hydroxyurea, an agent known to block DNA synthesis, was used to synchronize cells at the G_1/S boundary. Nocodozole, which interrupts microtubule formation, was used to synchronize cells in M phase. By analyzing transfected cells blocked by hydroxyurea, we could determine whether cells transfected with the p53 gene could progress to synchrony in G_1. In analyzing nocodazole-treated cells, we hoped to determine whether transfected cells could progress through G_1/S into the G_2/M block.

Saos-2 cells transfected with the mutant construct pC53SCX3 had a distribution within the cell cycle similar to that of the untransfected cells. When they were treated with hydroxyurea after transfection, both the transfected cells ($p53^+$) and the untransfected cells ($p53^-$) were synchronized prior to S phase (Fig. 6A). When the same cells were treated with nocodazole after transfection, most of the cells progressed through to synchrony in the G_2/M portion of the cell cycle (Fig. 6B). When Saos-2 cells were transiently transfected with the wild-type expression construct pC53SN3 and hydroxyurea was added, the cells expressing wild-type p53 were blocked at the same portion of the cell cycle as the untransfected cells (Fig. 6C). However, when the cells

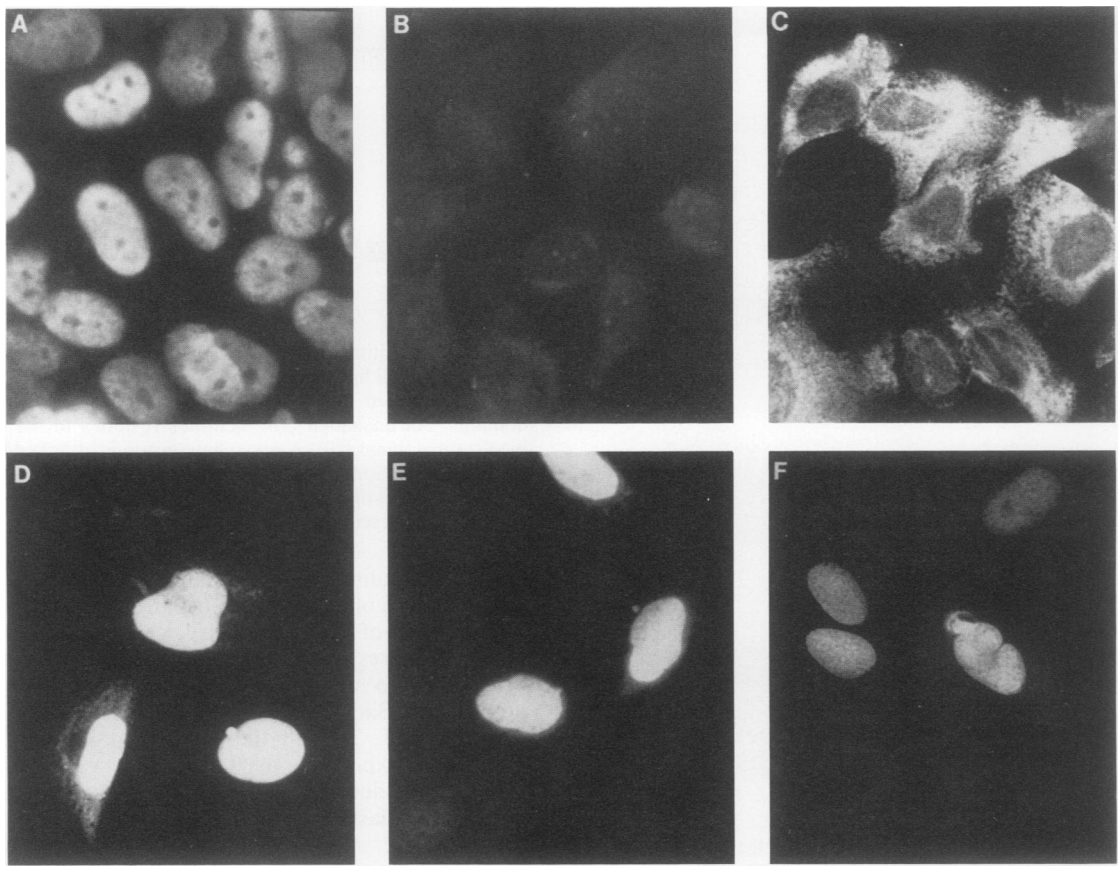

FIG. 5. Indirect immunofluorescence to identify subcellular localization of variant p53s. The primary antibody was PAb122, which recognizes p53. Secondary antibody was FITC-conjugated goat anti-mouse IgG. (A) Untransfected KHOS-240S cells. (B) Untransfected Saos-2 cells. (C) Saos-2 T512SN.1. (D) Saos-2 T718CX3.9. (E) Saos-2 cells transiently transfected with the wild-type p53 expression construct pC53SN3. (F) Saos-2 cells transfected with the mutant p53 expression construct pSVXR1.

containing wild-type p53 were treated with nocodazole, they were unable to progress through to the G_2/M block. The untransfected cells progressed as expected, showing a preponderance of blocked cells with 4N DNA content (Fig. 6D). As the cells that expressed wild-type p53 were unable to double their DNA content, it appears that overexpression of p53 blocks progression through the cell cycle in G_1 prior to S phase.

DISCUSSION

Because 9 of 10 independent osteosarcoma cell lines investigated in this study had detectable alterations in gene p53, the inactivation of this gene is likely to be important for the growth of many osteosarcomas. As this analysis was performed on established cell lines, it is possible that these geve p53 alterations occurred during propagation of the cells in vitro. Several previous observations make this unlikely. A similar frequency of gross rearrangements in the p53 gene has been noted for both primary tumors and established cell lines (40). Additionally, a high frequency of p53 gene alterations occurs in other primary tumors of the colon, lung, and breast (43, 46). Furthermore, transgenic mice that express mutant gene p53 in all their cells form osteosarcomas at a very high frequency (38). These experiments all support the hypothesis that loss of wild-type p53 is important for the actual formation and in vivo growth of osteosarcomas.

Whereas the introduction of the wild-type p53 gene suppressed the growth of osteosarcoma cells, introduction of the two mutant p53 expression constructs had no detectable effect on the growth of osteosarcoma cell lines. The mutant p53 genes were cloned from two carcinomas with single point mutations. This provides direct proof that subtle p53 mutations, such as the valine to alanine switch in the Cx-3 colon carcinoma that was present in the pC53SCX3 expression construct, inactivate the function of wild-type p53 as a growth suppressor. Reintroduction of p53 in osteosarcomas therefore provides an assay to determine whether p53 mutations alter its growth-suppressing function. As such, the transfection assay into osteosarcoma cells appears to provide a functional assay for p53.

Our present data indicate that the overexpression of the wild-type p53 gene severely limits the growth of the Saos-2, U-2OS, and KHOS-240S osteosarcoma cell lines. The growth suppression seen upon introduction of wild-type gene p53 into the Saos-2 and the KHOS-240S cells could be attributed to its reexpression. The fact that a similar growth suppression was seen upon introduction of a wild-type p53 gene into the U-2OS cells, which contain apparently wild-type endogenous p53, suggests that it is p53 overexpression that may suppress growth. As detected by radioimmunoassay, the level of p53 introduced by the expression constructs was several hundred-fold higher than the minute amount of wild-type p53 present in normal cells. The overexpression of

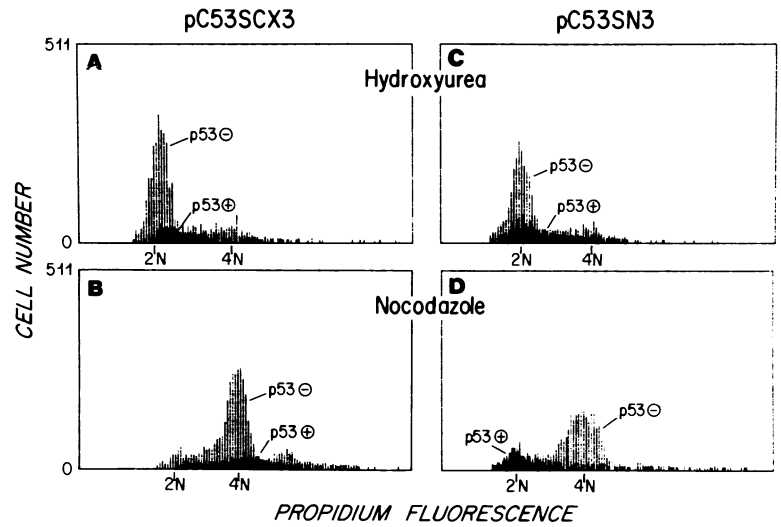

FIG. 6. Flow cytometric analysis of cell cycle control by p53. The relative number of cells from 0 to 511 is plotted against DNA content as measured by propidium iodide fluorescence and marked at the location of the diploid and tetraploid DNA content by 2N and 4N, respectively. Panels A and B represent Saos-2 cells transfected with the mutant p53 expression construct pC53CX3, and panels C and D represent Saos-2 cells transfected with the wild-type p53 expression construct pC53SN3. Panels A and C show cells that have been treated with hydroxyurea; panels B and D show cells treated with nocodazole. Untransfected cells are denoted by hatched bars labeled p53−, and transfected cells are denoted by solid bars labeled p53+. (In panel D, the hatched bars showing untransfected cells with 2N DNA content are almost completely obscured by the transfected cells' solid bar.)

wild-type p53 could represent nonspecific toxicity. This is quite unlikely, given the results of our cell cycle experiment, which shows that the effect of the wild-type protein is seen at a specific stage of the cell cycle, suggesting a regulatory rather than a toxic role for p53.

Because p53 is overexpressed in the transfected cells, it is not possible to infer that reestablishing physiologic p53 levels in osteosarcomas would cause a similar growth arrest. However, by overexpressing p53, it is possible to draw several conclusions about the role of this protein. Despite the numerous alterations that have accumulated within these established cell lines, including the absence of the retinoblastoma protein in most osteosarcomas (24) and aneuploidy in all, p53 is still capable of causing growth arrest. This indicates that the osteosarcoma cell lines maintain an intact pathway by which p53 can exert an effect on growth. Second, the overexpression of p53 is helpful in determining where in the cell cycle normal cell cycle control genes exert their effect. In the fission yeast *Schizosaccharomyces pombe*, both *suc1*$^+$ and *wee1*$^+$ were determined to be dose-dependent inhibitors of mitosis by the method of overexpression (30, 47). To determine that the normal c-*mos* product arrested *Xenopus* embryos in metaphase, it was also overexpressed (48). The cell cycle arrest by large quantities of wild-type p53 in G_1 in the osteosarcomas suggests that the normal function of p53 is as a cell cycle control protein. Indirect confirmation of this role comes from experiments in which altering the expression of the p53 gene appears to modulate the growth of colorectal carcinoma cells (2a).

In the past, several experiments that have investigated the effects of introducing p53 into cells have been done with similar constructs containing the SV40 promoter/enhancer which overexpress wild-type p53. The inability of E1a and *ras* to produce foci when cotransfected with constructs which overexpress p53 has been interpreted as suggesting that wild-type p53 is a suppressor of transformation (17, 20, 31). The inability to form foci might be due to a more general growth-arresting effect of p53 overexpression.

Why do normal cells contain wild-type p53? Overexpression of wild-type p53 blocks cell cycle progression in G_1 and results in inhibition of completion of S phase. Recent experiments have noted that p53 can inhibit SV40 DNA replication by inhibiting the binding of SV40 large T to polymerase alpha (27, 54, 59). It is suggested that wild-type p53 might also interfere with the binding of cellular initiation proteins to polymerase alpha (26). Wild-type p53 would therefore be expected to exert its effect prior to the completion of S phase. Our data are consistent with this model. p53 is underphosphorylated and expressed at slightly lower levels in G_0/G_1 prior to S phase (3). The potential overlap between the phase in the cell cycle at which overexpressed p53 blocks cell cycle progression and p53 is underphosphorylated suggests that it could be the underphosphorylated form of p53 that exerts a control within the cell cycle. This indicates that p53 might have to be phosphorylated in order to allow progress through the cell cycle. Recent work by Bischoff and co-workers has indicated that p34 is one of the kinases for p53 (3). A dynamic role for p53 between an active underphosphorylated p53 with growth-limiting function and an inactive phosphorylated p53 would provide a cell cycle-dependent growth suppression that is linked to the major known cell cycle control gene p34. A similar relationship has been postulated between the cell cycle phosphorylation and the activation and inactivation of $p105^{Rb}$, the product of the retinoblastoma gene (5, 6, 11). The cell cycle-specific growth suppression exerted by p53 and $p105^{Rb}$ is quite reminiscent of the check point control genes known to exist in *Saccharomyces cerevisiae* (29). The role of some of the tumor suppressor proteins, including p53 and the retinoblastoma gene product, might therefore be to monitor whether a cell should continue to progress through the cell cycle. Loss of this function would enable the cell to begin an escape from normal growth controls and propel it closer to the transformed phenotype.

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ADDENDUM IN PROOF

Mercer and co-workers have recently published results which show that p53 inhibits cell growth in a glioblastoma cell line (W. E. Mercer, M. T. Shields, M. Amin, G. J. Sauve, E. Appella, J. W. Romano, and S. J. Ullrich, 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).

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