Wild-Type p53 and a p53 Temperature-sensitive Mutant Suppress Human Soft Tissue Sarcoma by Enhancing Cell Cycle Control

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ABSTRACT

Soft-tissue sarcomas are a heterogeneous group of tumors that are putatively mesenchymal in origin. Therapeutic advances in this disease have been limited over the past several decades. Approximately one-half of all patients will ultimately succumb, usually to uncontrollable pulmonary metastases. Although little is known about the underlying molecular determinants driving soft-tissue sarcoma inception, proliferation, and metastasis, mutation of the p53 gene is the most frequently detected molecular alteration in this disease. Accordingly, we were interested in determining whether transduction of wild-type (wt) p53 into soft-tissue sarcomas bearing mutated p53 genes might alter the malignant phenotype. SKLMS-1 is a human-derived leiomyosarcoma cell line with a codon 245 p53 point mutation. Cationic liposome was used to transfected wt p53 or 143Ala temperature-sensitive mutant p53 into this cell line. SKLMS-1 stable transfectants expressing wt p53 had decreased cell proliferation in vitro, decreased in vitro colony formation in soft agar, and decreased tumorigenicity in severe combined immunodeficient mice in vivo. Flow cytometric analysis of cell cycle components demonstrated markedly increased G1 cell cycle arrest and decreased entry into S phase, which corresponded to the induction of p21cip1 protein in the transfectants. Using SKLMS-1 stable transfectants expressing the 143Ala p53 temperature-sensitive mutant, we demonstrated the kinetics of and the causal relationship between wt p53 expression, the wt p53-dependent induction of cell cycle inhibitor p21cip1, and inhibition of cell cycle progression in p53-transfected SKLMS-1 cells. The ability to restore wt p53 growth-regulatory functions in soft-tissue sarcoma may ultimately be useful as a future therapy in patients with soft-tissue sarcomas.

INTRODUCTION

Soft-tissue sarcoma is a heterogeneous cluster of malignancies that share a putative mesenchymal origin. This disease constitutes <1% of adult solid tumors, and the 5-year overall survival rate for this disease has stagnated at ~50% for the past several decades (1). Perhaps because of the relative rarity of soft-tissue sarcoma, it has been difficult to secure the human tumor tissues needed to gain insight into the molecular determinants underlying its proliferation and metastasis. The most common genetic mutation involves alteration in the p53 gene, a finding that is observed in 30–50% of adult soft-tissue sarcomas (2). Archival immunohistochemical studies in patients with known clinical outcome have demonstrated that p53 mutations are more frequent in metastatic than in primary sarcoma and in high-grade than in low-grade lesions (3). Moreover, patients with p53-mutated sarcoma have a markedly decreased overall survival relative to individuals whose sarcomas contain wt p53 (4).

In studies using autologous primary and metastatic synovial sarcoma, we have demonstrated that small subgroups of cells bearing p53 point mutation in the primary tumor expand and eventually predominate as the cellular components of the metastasis (5). This is particularly pertinent because of the recent demonstration that central tumor necrosis, a ubiquitous finding in soft-tissue sarcoma, creates a hypoxic environment that favors the survival of cells with diminished apoptotic potential because of their relative lack of wt p53 (6).

In light of these considerations, we have been interested in preclinical strategies designed to restore wt p53 in soft-tissue sarcomas bearing p53 mutations. Using SKLMS-1, a human-derived leiomyosarcoma bearing a p53 point mutation at codon 245 as recipient cell line, wt p53 expression vector was transfected by liposome-mediated gene transfer. Stable clones that produce wt p53 were established. In addition, stable transfectants containing a p53 temperature-sensitive mutant (143Ala) were also generated in SKLMS-1 such that the progeny have wt p53 at 32°C and mutated p53 at 38°C (7). These transfectants were used to demonstrate that the reintroduction of wt p53 in vitro inhibits SKLMS-1 proliferation and colony formation in soft agar. In addition, SKLMS-1 tumorigenicity in SCID mice was suppressed by the reintroduction of wt p53. The mechanisms underlying these alterations in the malignant phenotype appear to involve the reestablishment of cell cycle regulation, suggesting the possibility that restoration of wt p53 may have therapeutic applicability in soft-tissue sarcoma.

The abbreviations used are: wt, wild type; SCID, severe combined immunodeficiency; cdk, cyclin-dependent kinase.

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MATERIALS AND METHODS

Cell Culture. Human leiomyosarcoma SKLMS-1 cells from American Type Culture Collection were grown in DMEM/F12 medium with 10% fetal bovine serum and maintained at 37°C in an incubator containing 5% CO₂. wt p53-transfected cell clones were grown at 37°C. Temperature-sensitive transfectants were grown at 32°C for wt p53 and 38°C for mutant p53.

Plasmids and Stable Transfection. Human p53 expression vectors were generously provided by Dr. B. Vogelstein (Johns Hopkins University School of Medicine, Baltimore, MD). PCMV-neo-bam is an expression vector containing the cytomegalovirus long-terminal repeat promoter and the neomycin resistance gene under the control of the SV40 promoter. Either a wt or a mutated p53 gene was introduced into this vector to create PC53-SN3 (wt p53) or PC53-SCX3 (temperature-sensitive mutant 143Ala). The only difference between the two constructs is a single nucleotide (T to C) change, resulting in a substitution of alanine for valine at codon 143 in pc53-SCX3. All DNA transfections were carried out using liposome-mediated gene transfection. SKLMS-1 cells (2 x 10⁶ cells in a 100-mm dish) were transfected with a mixture of 15 µg of plasmid DNA and 225 µg liposome. Approximately 10 h after transfection, the cells were washed and cultured in fresh medium for 48 h and then split 1:10. The cells were cultured in a selection medium containing 800 µg/ml of G418 for 4-6 weeks. Individual G418-resistant colonies were cloned using cloning ring and expanded to mass culture.

PCR Analysis of Clones. The primers for PCR were: P1, 5'-CAATGGTTACTGAAGCCCAGC-3'; and P2, 5'-GGTGAAATTTCTCCATCCTGGT-3'. DNA was amplified following 30 cycles of PCR (1 min at 94°C, 1 min at 60°C, and 1 min at 72°C) using a Thermal Cycler 9600 (Perkin-Elmer Corp., Norwalk, CT). The PCR products were confirmed by electrophoresis. Those clones that proved positive were expanded for further analysis.

Southern Blot Analysis. For Southern blot analysis, 20 µg of DNA were digested with BamHI overnight, electrophoretically separated in 0.8% agarose gel, and blotted to nylon membranes. The membranes were prehybridized with 1× Church buffer at 65°C for 1 h and hybridized at 65°C overnight with a p53 cDNA probe labeled with [32P]dCTP using a random primer DNA labeling kit (Boehringer Mannheim Corp., Indianapolis, IN). Membranes were then washed and subjected to autoradiography.

Northern Blot Analysis. Total RNA was extracted by RNAzol B solution (Biotocos Laboratories, Inc., Houston, TX). For Northern blotting, 20 µg of total RNA were electrophoresed over a denaturing 1% agarose gel and blotted to nylon membranes. The membranes were prehybridized with 1× Church buffer at 65°C for 1 h and hybridized at 65°C overnight with a p53 cDNA probe labeled with [32P]dCTP using a random primer method of labeling, as described above. The membranes were then washed and subjected to autoradiography.

Western Blot Analysis. The cells were lysed by PBSD treatment buffer [154 mM NaCl, 10 mM Na₂HPO₄ (pH 7.2), 30 mM NaNO₃, 1 mM NaF, 12 mM Cs₂HPO₄, Na, 0.1% SDS, and 1% Triton X-100]. Protein (100 µg) was separated by SDS-PAGE (12% gel for p21cip, 7.5% gel for p53) and transferred onto a nitrocellulose membrane. The nitrocellulose membrane was blocked with 5% low-fat milk in PBST buffer (0.1% Tween 20 in PBS buffer) for 1 h. The membrane was probed with 1:200 diluted p21cip primary antibody (Santa Cruz Biotechnology, Inc.) or with 1 µg/ml of p53 Ab-6 primary antibody (Oncogene Science, Inc., Manhasset, NY) for 1 h at room temperature and washed with PBST buffer. Then, the membrane was probed with 1:2000 diluted horseradish peroxidase conjugated goat anti-rabbit IgG antibody or with 1:2000 diluted horseradish peroxidase conjugated goat anti-mouse IgG antibody for 1 h at room temperature. The blot was then washed in PBST buffer and detected with the enhanced chemiluminescence Western blotting detection reagents (Amersham Corp., Arlington Heights, IL).

Cell Growth Rate. The cells (3.5 x 10⁶ cells/well) were plated in 24-well plates in duplicate. The cells were harvested every day for 8 days and counted; their viability was determined by trypsin blue exclusion. The experiments were repeated twice.

Soft Agar Colony Formation Assay. The cells (1 x 10³ cells/well) were plated in a 24-well plate in culture medium containing 3.5% agarose overlaying a 0.7% agarose layer and incubated at 37°C for 5 weeks. The plates were then stained with 1 mg/ml of crystal violet (1 mg/ml) for 48 h at 37°C, after which the colonies were counted for each well and cell line. The experiment was set up as duplicates each time and repeated twice.

Tumorigenicity Assay. The cells were trypsinized, washed twice with PBS buffer, and centrifuged. The viable cells were counted; of those, 4 x 10⁶ cells were resuspended in 200 µl of Matrigel. Cell suspensions were injected s.c. into the right flank of 6-8-week-old SCID mice. Tumor volumes were estimated as the product of three-dimensional caliper measurements (longest surface length, width, and tumor thickness). The growth of the tumors was measured every week and monitored until the tumor size reached 2 cm³ (a minimum of 56 days and maximum of 150 days).

Flow Cytometry. The cells used for cytometry were trypsinized, washed three times with PBS, and centrifuged. The cells were fixed by the drop-wise addition of cold 70% ethanol and resuspended at 1 x 10⁶ cells/ml. The fixed cells were stored at 4°C for up to 2 weeks before analysis. For staining, the cells were washed with PBS and resuspended in 1 ml of PBS containing 50 µg/ml propidium iodide and 50 µg/ml RNase A. Analysis was performed using a Coulter EPICS profile flow cytometer (Coulter Corp., Hialeah, FL). Quantitation of the fraction of each cell cycle stage was performed with the MultiCycle DNA analysis program (Phoenix Flow System, San Diego, CA).

RESULTS

Establishment of Stable Transfectants Expressing wt p53 and 143Ala Temperature-sensitive Mutant. To determine whether restoration of wt p53 into p53 mutation-bearing human soft-tissue sarcoma could lead to tumor suppression, SKLMS-1, a human leiomyosarcoma cell line with a p53 point mutation at codon 245 (8), was transfected with the pC53-SN3 vector containing a neomycin-resistant selec-
tion marker gene. G418-resistant clones were selected and expanded into cell lines that were designated SKp53 cell lines. In addition, SKLMS-1 cells were also transfected with the pC53-SCX3 vector that expresses the 143Ala temperature-sensitive mutant of p53, and the progeny G418-resistant cell lines were named SKA1a cell lines. The same approach was used to select control SKneo cell lines, which are neomycin resistant but do not express wt p53.

It is possible that some of the SKp53 and SKA1a stable transfectants selected harbored only the neomycin resistance gene but not the wt p53 gene or 143Ala temperature-sensitive mutant of p53. To identify those SKp53 and SKA1a transfectants that integrated the transfected p53 genes, PCR experiments using primers specific for the transfected wt p53 gene or 143Ala temperature-sensitive mutant were performed with DNAs from the parental SKLMS-1, the control SKneo, and the SKp53 and SKA1a transfectants (Fig. 1A). An 825-bp PCR product representing the exogenous p53 gene was detected in 6 of 24 selected clones that were transfected with the wt p53 gene, whereas the 825-bp PCR product was detected at a higher frequency (12 of 24) in clones that were transfected with the 143Ala temperature-sensitive mutant of p53. The integration of the transfected p53 genes in the transfectants was further confirmed by Southern blot analysis using p53 cDNA probes, which demonstrated an additional exogenous p53 gene band of approximately 1.8 kb in the transfectants compared with the parental SKLMS-1 cells (Fig. 1B). To determine whether these SKp53 and SKA1a transfectants actually expressed p53 mRNA, Northern blot analysis with p53 cDNA probes was performed (Fig. 1C). Three of the SKp53 transfectants (SKp53-1, SKp53-2, and SKp53-3) and three of the SKA1a transfectants (SKA1a-1, SKA1a-2, and SKA1a-3) expressed exogenous p53 mRNA in addition to the endogenous mutated p53 mRNA. The parental SKLMS-1 and control SKneo cell lines, as expected, did not express exogenous p53 mRNA. It is notable that the SKA1a-1, SKA1a-2, and SKA1a-3 cells expressed much higher levels of exogenous p53 mRNA than the SKp53-1, SKp53-2, and SKp53-3 cells did. The relative amounts of exogenous p53 mRNA in SKp53-1, SKp53-2, SKp53-3, SKA1a-1, SKA1a-2, and SKA1a-3 transfectants were 3.6, 1.3, 1.7, 1.8, 1.7, and 1.4, respectively (Fig. 1), as determined by quantitation with the Personal Densitometer 5037 (Molecular Dynamics, Sunnyvale, CA). Equal loading of RNA was confirmed by Northern blot analysis on β-actin using β-actin probes (data not shown). We thus established a panel of stable transfectants that expressed different amounts of wt p53 or 143Ala temperature-sensitive mutants of p53. The SKLMS-1

**Fig. 1** PCR, Southern, and Northern analysis of the indicated cell lines and transfectants. In A, PCR amplification of a 0.8-kb fragment (Exo) corresponds to the entire coding region of p53 cDNA. The endogenous p53 gene shows a 1.8-kb fragment (Endo). In B, for Southern blot analysis, 20 μg of DNA were digested with BamHI and hybridized with a p53 cDNA probe. The exogenous p53 gene from the expression vector was present as a 1.8-kb fragment (Exo). The endogenous p53 gene was present in a 7.8-kb fragment (Endo). In C, for Northern blot analysis of p53 mRNA expression, 20 μg of total RNA were hybridized with a p53 cDNA probe. Exogenous p53 mRNA is seen at a position corresponding to a 2.6-kb fragment (Exo) and is differentiated from endogenous p53 mRNA of a 2.8-kb fragment (Endo).
and SKneo cells were used as control cell lines to make certain that the changes in biological properties in the p53 transfectants were not caused by the selection process, by transfection of the plasmids, or by the expression of the neomycin resistance gene.

**Suppression of Cell Growth and Tumorigenicity of SKLMS-1 Leiomyosarcoma Cells by p53.** Once the p53 transfectants expressing the wt p53 or 143Ala temperature-sensitive mutant of p53 were established, we first examined the effect of p53 restoration on the p53 mutant-bearing SKLMS-1 leiomyosarcoma cells *in vitro*, assessing growth properties and colony formation in soft agar. The growth rates of the SKp53-1, SKp53-2, and SKp53-3 were significantly inhibited compared with the parental SKLMS-1 and control SKneo cells beginning on the sixth day in culture (*P* < 0.01; Fig. 2). More striking growth inhibition was observed when SKA1a-1, SKA1a-2, and SKA1a-3 transfectants were cultured at 32°C with the wt p53 conformation than when cultured at 38°C with the mutant p53 conformation (*P* < 0.001; Fig. 3A). Although the parental SKLMS-1 cells cultured at 32°C grew slower than those cultured at 37°C (Fig. 3B), these cells were able to grow to confluency at 32°C, whereas growth of the SKA1a transfectants was stopped. These data indicate that the SKA1a transfectants were cytotstatic at 32°C mainly because of the effect of wt p53 at this temperature, not just the general effect of the lower temperature on cell proliferation.

Anchorage-independent growth in soft agar is an indicator of transforming ability in transformed cells such as human cancer cells. Transformed cells usually grow well in an anchorage-independent manner in soft agar, whereas non-transformed or less malignant cells usually cannot grow or grow poorly in soft agar. To test the impact of wt p53 on anchorage-independent growth, we assayed SKLMS-1 cells, SKneo cells, and SKp53 transfectants for their ability to grow in soft agar. The parental SKLMS-1 cells exhibited high efficiency in forming soft agar colonies, and the SKneo control cells also had relatively high efficiency in forming soft agar colonies (20% less than SKLMS-1 cells). In contrast, the colony-forming efficiency of the three SKp53 transfectants was reduced to 50% of the level of the SKLMS-1 cells (*P* < 0.01; Fig. 4A). More striking reductions in soft agar colony formation (60–70% reduction) were observed in SKA1a transfectants cultured at 32°C (wt p53 conformation) than at 38°C (mutant p53 conformation; *P* < 0.001; Fig. 4B). Whereas the parental SKLMS-1 cells cultured at 32°C showed less than 25% reductions of soft agar colony than those cultured at 38°C (Fig. 4B). This control experiment clearly demonstrated that reduced soft agar colony formation in SKA1a transfectants cultured at 32°C was mainly due to the effect of wt p53 at this temperature, not just the general effect of the lower temperature. Taken together, these data indicate that restoration of wt p53 can inhibit cell proliferation and anchorage-independent growth of SKLMS-1 human leiomyosarcoma cells.

A critical test for the effect of p53 restoration on SKLMS-1 cells is whether such restoration can suppress tumor formation *in vivo*. Therefore, tumorigenicity assays were performed in SCID mice that were injected s.c. with 4 × 10⁶ cells of either the parental SKLMS-1, the control SKneo, or one of the three wt p53-expressing SKp53 transfectants (Fig. 5). Rapid tumor growth became detectable 2 weeks after the inoculation of...
SKLMS-1 and SKneo cells, which developed into large tumors (681 ± 127 mm$^3$) by 6 weeks after injection and huge tumors (3510 ± 794 mm$^3$) by 8 weeks after injection (Fig. 5). In contrast, tumors in mice injected with the same number of any of the three SKp53 transfectants were only 151.5 ± 15.9 mm$^3$ by 8 weeks after injection (Fig. 5). The tumor-suppressing function of wt p53 was more dramatic in mice injected with the SKp53-1 and SKp53-2 transfectants, which did not induce tumor until 7–8 weeks after injection. Overall, inhibition of tumor growth in mice injected with the three SKp53 transfectants was highly significant ($P < 0.01$) compared with tumor growth in mice injected with either the parental SKLMS-1 or the control SKneo cells. These results clearly demonstrate that wt p53 can suppress the tumorigenic potential of SKLMS-1 human leiomyosarcoma.

**Effect of wt p53 Expression on Cell Cycle Progression.** The striking differences observed between SKLMS-1 cells and SKp53 and SKA1a transfectants regarding *in vitro* proliferation, soft agar colony formation, and *in vivo* tumorigenicity prompted us to further investigate the underlying mechanisms. Because p53 is a key regulator of the cell cycle, we investigated the effect of reintroducing wt p53 on cell cycle progression in the transition from a resting to a growing phase. Because the three SKA1a transfectants expressed higher levels of transfected p53 mRNA and had more dramatic effects on *in vitro* proliferation and soft agar colony formation, we performed flow cytometric analyses of cell cycle progression in the three SKA1a transfectants cultured at 32°C and at 38°C for 4 days using SKLMS-1 cells as control. The three SKA1a transfectants grown at 32°C demonstrated a dramatic increase in cells arrested at the G1 phase of the cell cycle than those grown at 38°C ($P < 0.005$), and there was a concomitant significant decrease ($P < 0.001$) of the SKA1a transfectants in the S phase of the cell cycle in cells grown at 32°C compared with those grown at 38°C (Table 1). In contrast, the SKLMS-1 cells grown at 32°C demonstrated a noticeable decrease in cells arrested at the G1 phase of cell cycle than in those grown at 38°C (Table 1).

The $p21^{cip}$ gene has been identified as a wt p53-inducible gene (9) that encodes a Mr 21,000 protein and functions as an inhibitor of cdk (10). The $p21^{cip}$ inhibits the growth of both human tumor cell lines (9) and normal diploid fibroblasts (10). Because $p21^{cip}$ is induced by DNA-damaging agents, leading to G1 arrest or apoptosis except in cells with mutant p53 (9), the $p21^{cip}$ gene product likely mediates the p53 function downstream of p53 (9–11). Accordingly, it was of interest to examine whether the increase in SKA1a transfectants arrested at the G1 phase of the cell cycle at 32°C correlated with the induction of $p21^{cip}$ cdk inhibitor. Western blot analysis demonstrated a dramatic induction of $p21^{cip}$ protein in the SKA1a transfectants at 32°C (wt p53 conformation) compared with that seen at 38°C (mutated p53 conformation; Fig. 6). These results suggest that one plausible mechanism underlying the growth inhibition mediated by the reintroduction of wt p53 was that p53 inhibited cell cycle progression from G1 to S and G2-M phases, which temporally correlated with the induction of the $p21^{cip}$ cdk inhibitor.

**Establishing the Causal Relationship and Specificity of Growth Inhibition by wt p53.** Although the above studies suggested a relationship between wt p53 expression, $p21^{cip}$ induction, and G1 cell cycle arrest, it was important to determine whether there was a direct causal relationship between these factors. To ensure that the increase of SKA1a cells in the G1 phase at 32°C was indeed due to the effects of induction of $p21^{cip}$ by wt p53, we examined whether G1 cell cycle arrest and $p21^{cip}$ induction were reversible when the wt p53 in the SKA1a-1 cells was removed by switching their growing temperature from 32°C to 38°C. SKA1a-1 cells were examined for cell cycle distribution by flow cytometry on days 0, 2, 4, and 6 after...
the temperature was switched to 38°C (Fig. 7, top panels). SKA1a-1 cells that were switched from 32°C to 38°C underwent a 30% decrease in cells in the G1 phase (day 0: 66.1% versus day 6: 46.9%; P < 0.001) as well as a concomitant significant increase (P < 0.001) in the percentage of cells in the S phase (day 0: 8.2% versus day 6: 25.2%). Western blot analyses using antibodies against p21cip1 and p53 were performed on SKA1a-1 cell lysates prepared at the same time points (Fig. 8). A discernible reduction in p21cip1 protein was observed after 4 days, and the loss of p21cip1 expression was observed 6 days after the temperature was switched to 38°C, whereas the p53 protein level increased 2 days after the temperature switch, indicating the appearance of mutated p53 proteins which have a longer half-life.

We also examined whether G1 cell cycle arrest and p21cip1 expression were inducible when wt p53 was made by switching the culture temperature of SKA1a-1 cells from 38°C to 32°C. As described above, experimental assessments were conducted at 0, 2, 4, and 6 days after the temperature was switched to 32°C. The percentage of SKA1a-1 cells in the G1 phase increased from 47.0% on day 0 to 57.2% by day 4 (P < 0.001), whereas the percentage of cells in the S phase decreased from 36.1% on day 0 to 11.1% by day 6 (P < 0.001; Fig. 7, bottom panels). The induction of p21cip1 (Fig. 8A) and wt p53 proteins (Fig. 8B) paralleled the G1 cell cycle arrest that was demonstrated by flow cytometric analysis (Fig. 7). Similar results were also observed using SKA1a-2 and SKA1a-3 cells (data not shown). We also considered the possibility that wt p53 restoration might be suppressing cell growth via induction of apoptosis. However, we did not observe apoptotic morphological changes, and DNA fragmentation by ladder assay and terminal deoxynucleotidyl transferase-mediated nick end labeling assay in either SKp53 or SKA1a transfectants cultured at 32°C 7 days after serum starvation (data not shown), indicating no demonstrable induction of apoptosis in SKLMS-1 cells by wt p53 under these conditions. Taken together, our data suggest that a major mechanism underlying the G1 arrest and growth inhibition of SKA1a cells at 32°C is due to the induction of the cell cycle inhibitor p21cip1 by wt p53.

**DISCUSSION**

The above studies demonstrate that restoration of wt p53 into p53-mutated soft-tissue sarcoma inhibits *in vitro* cell pro-
Table I  Percentage of cells in different phases of the cell cycle determined by flow cytometry

<table>
<thead>
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<th>Cell line/ Temperature</th>
<th>% G1 phase</th>
<th>% G2 phase</th>
<th>% S phase</th>
</tr>
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<tbody>
<tr>
<td>SKLMS-1/32°C</td>
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<td>21.9</td>
<td>37.6</td>
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<td>67.4</td>
<td>11.3</td>
<td>21.3</td>
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<td>66.1</td>
<td>21.6</td>
<td>8.2</td>
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<td>SKA1a-3/38°C</td>
<td>42.2</td>
<td>21.8</td>
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Fig. 5  Reintroduction of wt p53 inhibited soft-tissue sarcoma tumor formation. Cells (4 × 10⁶) from SKLMS-1 parental cells, SKneo control cells, or one of three SKp53 transfectants were injected s.c. into the right flank of 6- to 8-week-old SCID mice. Tumor volumes were measured by caliper in three orthogonal diameters. Five mice were tested for each cell line. The tumor growth was measured every week until it reached 2 cm³ (range, 56 to 150 days). The tumor volumes were presented as mean values; bars, SE.

Fig. 6 Western blot analysis of p21<sup>wt</sup> in temperature-sensitive transfectants. SKA1a-1, SKA1a-2, and SKA1a-3 cells were grown at 32°C or 38°C for 5 days. The cells were lysed, and 200 µg of protein were separated on a 12% SDS-PAGE gel. Anti-p21<sup>wt</sup> polyclonal antibody was used to detect p21<sup>wt</sup> expression. Equal loading of protein lysates was confirmed by Western blot analysis on α-actin using Ab-1 anti-α-actin monoclonal antibody (data not shown). The arrow indicates the expected location of p21<sup>wt</sup> protein.

Several issues raised by the above results merit further discussion. It is interesting that there were considerable differences in the extent to which the malignant phenotype was suppressed in the SKp53 stable transfectants compared with SKA1a transfectants cultured at 32°C. The in vitro inhibition of cell proliferation was much greater in the SKA1a temperature-sensitive mutants at 32°C than in the SKp53 stable transfectants. This difference was not due to the 32°C temperature per se, which inhibited proliferation of the SKLMS-1 parental cells, but not nearly to the extent observed in the SKA1a transfectants. In a similar manner, induction of the p21 protein and the percentage of cells arrested in the G1 phase of the cell cycle were not as great in SKp53 as they were in SKA1a transfectants at 32°C.

A possible explanation for these differences may be that the SKp53 transfectants contained significantly less exogenously transfected p53 mRNA than did the SKA1a transfect
A. 32°C to 38°C

Day 0

G1: 66.1
S: 8.2
G2/M: 21.5

Day 2

G1: 61.9
S: 10.6
G2/M: 24.8

Day 4

G1: 53.4
S: 20.1
G2/M: 24.2

Day 6

G1: 46.9
S: 25.2
G2/M: 25.8

B. 38°C to 32°C

Day 0

G1: 47.0
S: 36.1
G2/M: 15.1

Day 2

G1: 53.6
S: 26.8
G2/M: 17.5

Day 4

G1: 57.2
S: 14.3
G2/M: 26.7

Day 6

G1: 52.3
S: 11.1
G2/M: 33.5

Fig. 7  Cell cycle analysis of the SKA1a-1 temperature-sensitive transfectant after a temperature shift. SKA1a-1 cells were harvested after incubation for 0, 2, 4, and 6 days by switching their growing temperature from 32°C to 38°C or from 38°C to 32°C as indicated. The cells were stained with 50 μg/ml of propidium iodide in PBS containing 50 μg/ml RNase. Analyses were performed using a Coulter EPICS profile flow cytometer.

Fig. 8  Western blot analysis of p21 and p53 expression in SKA1a-1 temperature-sensitive transfectants after a temperature shift. After a switch in temperature from 32°C to 38°C or from 38°C to 32°C as indicated, SKA1a-1 cells were harvested and lysated after 0, 2, 4, and 6 days incubation. One hundred μg of protein were separated by SDS-PAGE (12% gel for p21 protein, 7.5% gel for p53). In A, anti-p21 polyclonal antibody was used to detect p21 protein. In B, p53 Ab-6 monoclonal antibody was used to detect p53 protein expression. Equal loading of protein lysates was confirmed by Western blot analysis on α-actin using Ab-1 anti-α-actin monoclonal antibody (data not shown). Experiments were repeated twice. The arrows indicate the expected location of p21 and p53 protein.

In these studies, only 1 of 35 MDA-MB468 clones and 8 of 55 T47D clones were positive for wt p53, suggesting the extreme efficacy of wt p53 in the suppression of tumor cell proliferation. Similar suppression of tumor proliferation was reported by Dittmer et al. (16), who observed a greater than 90% reduction in the plating efficiency of three murine tumor cell lines after transfection with wt p53. We have experienced a lower efficiency in establishing SKp53 transfectants (6 of 24) than in establishing SKA1a transfectants (12 of 24). Moreover, the SKA1a transfectants were selected at 37°C when the 143Ala was in the mutant conformation. These results imply that p53 expression was growth suppressive and that transfectants expressing higher levels of wt p53 did not survive.

It is notable that the in vivo tumor suppression effect of SKp53 transfectants was more dramatic than their in vitro cell proliferation inhibition. The differences imply that wt p53 may regulate certain factors that render the host environment unfavorable for tumor cell growth in vivo. One of the possible explanations is that wt p53 may inhibit tumor angiogenesis by down-regulation of the expression of vascular endothelial growth factors or other angiogenic factors (17). Presently, we are performing a systematic investigation on this additional p53 tumor-suppressing mechanism.

The temperature-sensitive 143Ala mutants were first described by Zhang et al. (7), who demonstrated that p53-mediated DNA binding and transcriptional activation occurred at 32°C but not 37.5°C using the K562 human leukemia cell line transfected with 143Ala. At 32°C, 143Ala only weakly inhibited K562 cellular proliferation. In contrast, 143Ala transfected into SKLMS-1 resulted in dramatic inhibition of cellular proliferation when cultured at 32°C compared with culturing at 38°C or compared with SKp53 transfectants. The differences in the
relative efficacy of 143Ala in inhibiting proliferation in K562 versus SKLMS-1 may be explained by the demonstrable lack of endogenous p53 mRNA in K562 cells (7) compared with parental SKLMS-1 cells, where endogenous mutated p53 mRNA could be readily detected. The possible gain of proliferative function driven by the presence of mutated p53 in parental SKLMS-1 (in contrast to the lack of endogenous p53 in K562) may have created a larger “window” for the subsequent inhibition of proliferation in SKAAl at 32°C.

The temperature switching experiments on the SKAAl transfectants (32°C to 38°C, or 38°C to 32°C) suggest that the wt p53 growth-inhibitory effect follows a relatively rapid time course. The timing and mechanism targeted by a given wt p53 restorative intervention may be critical to the future clinical efficacy of such measures. Other considerations, such as the effects of dose response, will also be relevant to future therapeutic applications. It has been suggested that wt p53 may be dominant over mutant p53 in certain two-allele configurations such as Saos-2 cells, where even 10-fold less exogenous single copy wt p53 could suppress the mutant p53-mediated neoplastic phenotype (18). Our data from SKp53 transfectants clearly demonstrate that even low levels of wt p53 are sufficient to inhibit tumor formation by SKp53 cells (Fig. 5). In contrast, higher wt p53 doses in SKAAl exerted a more striking growth-inhibitory effect that the SKp53 cells could not match (Figs. 2 and 3). However, the efficacy of this experimental system must be tempered by the reality of p53 gene mutational heterogeneity in soft-tissue sarcoma as borne in human patients (5). In this latter arena, additional considerations such as the feasibility of p53 gene delivery to the tumor, penetration into the tumor, sustained expression in tumor cells, and the subsequent inducibility of beneficial therapeutic effects may all be critical.

To test our findings in vivo, we have recently developed an isolated limb perfusion model in the sarcoma-bearing nude rat (19). This regional approach allows for the delivery of very high gradients of biochemotherapy directly to tumor that is not feasible using standard systemic drug delivery systems (20). In this model, it has been possible to successfully and selectively introduce both the LacZ reporter gene and p53-FLAG vectors into the sarcoma, and efficacy and dose-response studies are presently being performed. Such novel gene delivery systems may be particularly useful in delivering growth-suppressing constructs to cells bearing p53 mutations, such as wt p53 expression vectors, the recently described p53-derived synthetic peptides (21, 22), mutant p53-binding monoclonal antibodies (23), or other genes such as p16, (24) which can also inhibit the mutant p53-mediated malignant phenotype. It is hoped that such future interventions will prove to be effective in patients burdened by all-too-frequently lethal soft-tissue sarcoma.

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