

Wild-Type p53 Gene Transfer Inhibits Invasion and Reduces Matrix Metalloproteinase-2 Levels in p53-Mutated Human Melanoma Cells

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The tumor suppressor gene p53 has inhibitory effects on cell growth and angiogenesis and induces apoptosis when overexpressed in melanoma and in a variety of tumor cells by adenovirus-mediated gene transfer. The invasive ability of tumor cells, facilitating local infiltration and metastasis, is related to matrix metalloproteinase levels. In melanoma, matrix metalloproteinase-2 and matrix metalloproteinase-9 have a prominent role in this process. The aim of this study was to evaluate whether wild-type p53 overexpression, obtained by a recombinant adenovirus vector (AdCMV.p53), affects cell invasiveness through modulation of matrix metalloproteinase-2 and matrix metalloproteinase-9. Two human melanoma cell lines were used in this study: the SK-MEL-110, carrying a mutated p53 gene, and the SK-MEL-147, carrying the wild-type p53 gene. SK-MEL-110 cells infected with AdCMV.p53 exhibited decreased invasion capability from day 1 after infection, compared with cells not infected or infected with the control vector AdCMV.Null. This

reduced invasiveness was associated with decreased matrix metalloproteinase-2 levels in conditioned media whereas no changes were detected in matrix metalloproteinase-9 secreted levels. No modulation in matrix metalloproteinase-2 mRNA levels was detectable, however, after wild-type p53 gene transfer. Furthermore, protein expression of secreted tissue inhibitor of metalloproteinase-2 was not altered by AdCMV.p53 treatment. In contrast, in SK-MEL-147 cells, AdCMV.p53 did not affect cell invasiveness and levels of secreted matrix metalloproteinase-2. Gene transfer of wild-type p53 inhibited proliferation of both cell lines, showing that also SK-MEL-147 cells respond to wild-type p53 overexpression. This novel mechanism of action of wild-type p53 gene transfer may contribute to its antitumor effect by downregulating cell invasion and matrix metalloproteinase-2 secreted levels in mutated p53 human melanoma cell lines. **Key words:** gene therapy/recombinant adenovirus/tumor invasiveness/tumor suppressor genes. *J Invest Dermatol* 114:1188-1194, 2000

Despite extensive research the incidence of malignant melanoma is increasing in the Western world and the prognosis remains extremely poor for patients with metastatic disease (Rigel *et al*, 1996; Demierre and Koh, 1997; Legha, 1997). The spreading process of melanoma metastasis involves a series of complex steps; among them the extracellular matrix (ECM) degradation plays a prominent role allowing tumor cell invasion and metastasis formation. Previous data from our and other laboratories have demonstrated that gene transfer strategies, aimed at overexpressing wild-type (wt) p53 by viral vectors, exhibit a therapeutic potential *in vivo* by inhibiting tumor growth and metastasis in melanoma and in a variety of other malignancies (Fujiwara *et al*, 1994; Cirielli *et al*, 1995; Clayman *et al*, 1995; Harris *et al*, 1995; Leeson-Wood *et al*, 1995; Yang *et al*, 1995). The primary function of wt p53 protein in normal mammalian cells is the maintenance of DNA integrity and

genomic stability implemented by cell cycle arrest allowing the cells to repair damaged DNA (Kastan *et al*, 1992; Kuerbitz *et al*, 1992; Livingstone *et al*, 1992; Yin *et al*, 1992; Perry and Levine, 1993). The second prominent function of p53 is the execution of programmed cell death, i.e., apoptosis (Lane, 1992).

Inactivation of the p53 gene product after missense mutations and its interaction with oncogenic viral or cellular proteins leads to a selective growth advantage, generally observed as tumor progression (Baker *et al*, 1989; Momand *et al*, 1992), whereas replacement of the normal p53 gene in p53-defective tumor cells suppresses tumorigenicity *in vivo* (Donehower, 1996).

Phase 1 trial results obtained from patients with advanced nonsmall cell lung cancer and recurrent head and neck cancers demonstrate the feasibility and safety of wt p53 gene therapy using retroviral or adenoviral vectors, and its efficacy in inducing tumor regression or disease stabilization in these patients (Roth *et al*, 1996; 1998; Clayman *et al*, 1998; Schuler *et al*, 1998; Swisher *et al*, 1999).

The therapeutic effect of wt p53 overexpression in cancers, observed both *in vitro* and *in vivo*, has been related to its inhibitory action on cell proliferation and to the induction of apoptosis (Yonish-Rouach *et al*, 1991; Fujiwara *et al*, 1993; Cirielli *et al*, 1995; Yang *et al*, 1995; Zhang *et al*, 1995). Furthermore, it has been shown that wt p53 overexpression has an antiangiogenic effect *in*

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in vivo (Riccioni *et al*, 1998). This additional antitumor mechanism may be due to thrombospondin induction (Van Meir *et al*, 1994), basic fibroblast growth factor downregulation (Ueba *et al*, 1994), or a direct effect towards the inhibition of endothelial cell differentiation (Riccioni *et al*, 1998).

As p53 inactivation has been shown to increase invasive ability of lung carcinoma cells *in vitro* (Mukhopadhyay and Roth, 1996) and to correlate with basement membrane degradation in superficial urothelial carcinomas (Ozdemir *et al*, 1997), it may be speculated that control of the mechanisms modulating cell invasion can also underlie part of the wt p53 antineoplastic effect. Basement membrane and extracellular matrix degradation, which are the first steps required for cell invasion, are regulated by the matrix metalloproteinases family (MMPs), a class of zinc-dependent endopeptidases playing a key role in tumor cell invasion and metastasis (Ray and Stetler-Stevenson, 1994). Among this large family of proteinases, MMP-2 and MMP-9, the 72 kDa and 92 kDa gelatinases, are specifically involved in type IV collagen degradation, and have been shown to be actively secreted *in vitro* by a variety of melanoma cells (Hujanen *et al*, 1994; Ray and Stetler-Stevenson, 1995). Specifically, MMP-2 is detected in early stages of melanocytic tumor progression. In fact, it is expressed in junctional nests of benign nevi, and its expression directly associates with atypia, suggesting that it may be of prognostic value in metastatic melanoma behavior (Väisänen *et al*, 1996). Furthermore, both interleukin-8 and vitronectin upregulation of MMP-2 expression in melanoma cells correlated with both increased invasiveness *in vitro* and metastatic potential *in vivo* (Luca *et al*, 1997; Bafetti *et al*, 1998).

Based on these reports we hypothesized that, in addition to its known pro-apoptotic and antiangiogenic effects, overexpression of wt p53 may inhibit melanoma cell invasiveness, and that this effect may be associated with a modulation of MMP-2 or MMP-9 production. Mutations in the p53 gene are among the most frequent molecular alterations in human cancers (Levine *et al*, 1995) but appear less frequently in melanoma (Albino *et al*, 1994; Lübke *et al*, 1994; Weiss *et al*, 1995; Halaban, 1996; Hartmann *et al*, 1996). Although p53 is not a key player in melanoma pathogenesis, mutant p53 gene may confer a survival advantage for melanoma cells contributing to the growth of metastatic cells (van Golen *et al*, 1996). For this reason and despite the low frequency of p53 mutations in this study we investigated whether adenovirus (Ad) mediated wt p53 gene transfer may have biologically relevant effects on cell invasion and MMP-2 and MMP-9 protein expression in both wt and mutant p53 expressing melanoma cell lines.

MATERIALS AND METHODS

Cell lines, Ad infection, and cell number assessment In this study we used two different human melanoma cell lines, SK-MEL-110 and SK-MEL-147, which carry mutated and wt p53, respectively (Albino *et al*, 1994). Cells were grown in Dulbecco's modification of Eagle's medium (DMEM; Gibco BRL, Gaithersburg, MD) supplemented with 10% fetal bovine serum (Gibco BRL). Recombinant, replication-deficient adenoviral vectors, AdCMV.p53 expressing human wt p53 (a generous gift from Dr. B. Vogelstein, Baltimore, MD) and AdCMV.Null as control vector with no insert in the expression cassette, were amplified and titrated in 293 cells as previously described (Graham and Prevec, 1995). Infections were carried out overlaying cells with adenoviral suspensions in serum-free DMEM for 1 h 30 min at 37°C (Cirielli *et al*, 1995; Yang *et al*, 1995). In preliminary experiments infection efficacy on SK-MEL-110 and SK-MEL-147 cells was determined with an Ad vector expressing nuclear-localized β -galactosidase (AdCMV.NLS β -gal). Cells were infected with 10, 20, and 50 plaque-forming units (pfu) per cell and cultured for 48 h. Following fixation and incubation with 1 mg per ml X-gal for 1–4 h to detect β -galactosidase activity (Cirielli *et al*, 1995; Yang *et al*, 1995), the percentage of positive (blue) cells was determined and it was found to be similar between the two cell lines.

Cell number was assessed by direct cell counts, and Trypan blue dye exclusion assay was used as an indicator of cell viability.

Western blot analysis for p53 and tissue inhibitor of metalloproteinase-2 (TIMP-2) For p53 protein detection, cells were lysed in a

modified RIPA buffer (150 mM NaCl, 50 mM tri(hydroxymethyl)-aminomethane (Tris) at pH 7.5, 1% NP40, 0.25% sodium deoxycholate, 1 mM sodium orthovanadate, 50 μ g per ml aprotinin, 50 μ g per ml leupeptin, 1 mM phenylmethylsulfonyl fluoride, 2 mM ethyleneglycol-bis(β -aminoethyl ether)-N,N,N'-tetraacetic acid). Cells were incubated in RIPA buffer for 30 min on ice and subjected to three rounds of freezing and thawing at the end of the incubation. Conditioned media from SK-MEL-110 cells were used for the detection of TIMP-2. Protein concentrations were determined by a Bradford assay (Bio-Rad, Hercules, CA) using bovine serum albumin (BSA) as standard. Twenty microgram samples (total protein content) of total cell lysates for p53 detection and 10 μ g samples of conditioned media for TIMP-2 detection were run in 8% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were then transferred to a nitrocellulose filter (Hybond, Amersham, Chicago, IL) at 30 V overnight. The filters were then blocked in blocking buffer containing 5% nonfat dry milk and 0.1% Tween-20 in phosphate-buffered saline (PBS) for 1 h at room temperature and probed with the mouse monoclonal antihuman p53 IgG antibody DO-1 (Santa Cruz Biotechnology, Santa Cruz, CA), or with the mouse monoclonal antihuman TIMP-2 IgG antibody Ab-1 (Oncogene Research Products, Cambridge, MA) for 1 h. Membranes were washed in PBS/0.1% Tween-20 and incubated with secondary antibody, horseradish-peroxidase-conjugated goat antimouse IgG (Amersham), for 30 min. Membranes were then washed in PBS/0.1% Tween-20 and developed using the enhanced chemiluminescence kit (Amersham), according to the manufacturer's instructions.

Gelatin zymography Gelatin zymographies were used to visualize the type IV collagenases (MMP-2 and MMP-9).

Culture supernatants were collected, concentrated by Centricon (3 kDa cut-off) (Amicon, Beverly, MA) and analyzed for collagenase activity. Total protein content was measured by the Bradford method (Bio-Rad, Hercules, CA). Two micrograms of proteins were diluted into the sample buffer (0.4 M Tris at pH 6.8, 5% glycerol, 0.03% bromophenol blue) and electrophoresed onto 9% polyacrylamide gels containing SDS and embedded with 1 mg per ml gelatin (Sigma Chemical, St. Louis, MO). After electrophoresis, gels were incubated for 1 h in 2.5% Triton X-100 and for additional 18 h at 37°C in 50 mM Tris-HCl at pH 7.5, 0.2 M NaCl, 5 mM CaCl₂, and 0.02% Brji 35. At the end of the incubation, the gels were stained with 0.5% Coomassie G 250 (Bio-Rad, Richmond, CA) in methanol:acetic acid:H₂O (30:10:60). To quantitatively compare the lysis signals, the gels were analyzed with a densitometer (GS-700 Imaging; Bio-Rad) as previously described (Kleiner and Stetler-Stevenson, 1994).

Chemoinvasion assay To assess cell invasion, the Boyden chamber chemoinvasion assay was carried out as previously described (Albini *et al*, 1987). Briefly, polycarbonate filters (8 μ m pores, PVP-free, Nucleopore Costar Scientific, Cambridge, MA) were coated with reconstituted basement membrane proteins (Matrigel, Collaborative Research, Waltham, MA), which was dried and reconstituted. Filters were rehydrated with DMEM before use and NIH-3T3-conditioned medium was used as chemoattractant. Serum-free DMEM containing 0.1% BSA was used as negative control. Cells in Petri dishes were washed twice with PBS to eliminate any floating cells, harvested with trypsin, and washed with serum-free DMEM containing 0.1% BSA. Then, cells were placed in the upper compartment of the Boyden chamber (1×10^5 cells per chamber). Cell number was assessed by direct cell count. Trypan blue dye exclusion assay demonstrated that at least 99.5% of the harvested cells were viable. Boyden chambers were incubated at 37°C in 5% CO₂/95% air for 6 h. Cells remaining on the upper surface of the filters were mechanically removed; those which migrated to the under surface were fixed with ethanol, stained with toluidine blue for at least 10 min and counted under 400 \times magnification. Ten random fields per slide were counted. Assays were performed in triplicate and repeated at least four times in independent experiments.

Chemotaxis assay To determine cell migration, chemotaxis assays were performed in the same manner as for chemoinvasion; however, filters were coated with 5 μ g per ml of gelatin alone, without Matrigel. Assays were performed in triplicate and repeated three times in independent experiments.

RNA analysis Total cellular RNA was isolated by the acid guanidium thiocyanate phenol chloroform method (Chomczynski and Sacchi, 1987). After determination of RNA purity and concentration, 20–25 μ g of total RNA was fractionated using 1% agarose gels containing formaldehyde. RNA was transferred overnight onto nylon membrane (Amersham) and then immobilized by ultraviolet irradiation. Prehybridation and hybridation of the membranes was accomplished in QuikHyb hybridation solution

(Stratagene). The cDNA fragment of human MMP-2 (Levy *et al*, 1991) (kindly provided by W.G. Stetler-Stevenson) was labeled with $\alpha^{32}\text{P}$ dCTP by the random-primer method. After incubation the blots were washed in $2\times$ standard saline citrate (SSC) with 1% SDS for 15 min at room temperature, then in $2\times$ SSC with 1% SDS for 30 min at 60°C , and finally were exposed to Biomax (Biomax Kodak, Rochester, NY) at -80°C . Autoradiograms were scanned by Gel Doc1000 apparatus. The signal strength of MMPs RNA was normalized for each sample with respect to the density of the corresponding glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA signal, to compensate for variation in RNA loading and transfer.

Statistical analysis All data are expressed as mean \pm standard error of the mean (SEM). Statistical analysis was performed by analysis of variance (ANOVA) followed by Student-Newman-Keuls *posthoc* test when appropriate. A value of $p < 0.05$ was considered statistically significant.

RESULTS

Infection efficacy and p53 gene transduction To assess the infection efficacy of adenoviral vectors both cell lines were infected with AdCMV.LacZ at 10, 20, or 50 pfu per cell. Infection efficacy, evaluated as the percentage of β -galactosidase-expressing cells, was greater than 95% for both SK-MEL-110 and SK-MEL-147 cells

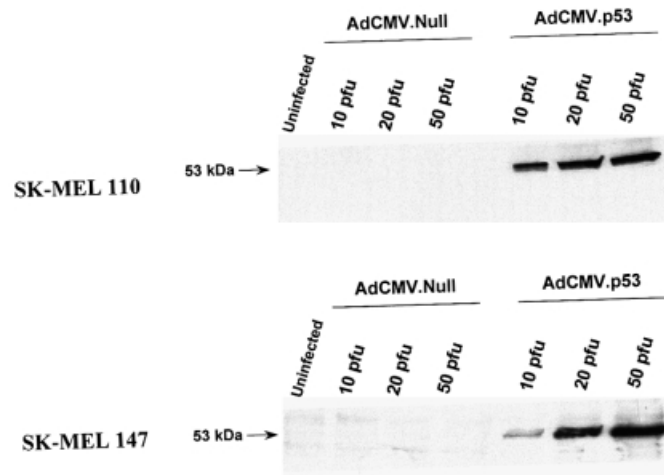


Figure 1. Expression of wild type p53 in cell lysates 1 d after infection with AdCMV.p53. Western blot analysis was performed on SK-MEL-110 and SK-MEL-147 cell lysates. Detectable levels of wt p53 are found only in AdCMV.p53-treated cells, increasing with vector concentration (10, 20, or 50 pfu per cell). Similar results were obtained in two separate experiments.

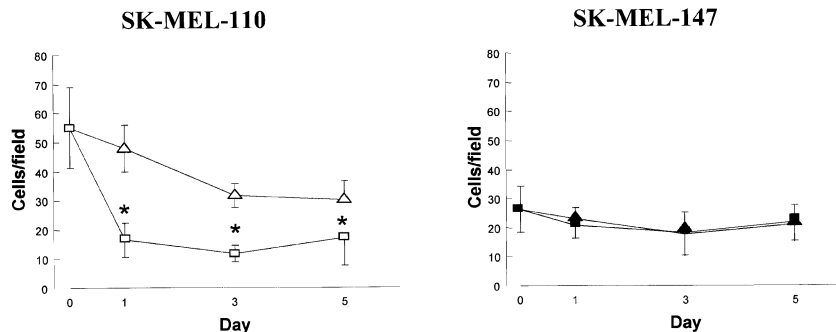


Figure 2. Cell invasion through Matrigel. To evaluate cell invasion, chemoinvasion assay was performed using a modified Boyden chamber. The number of cells attached to the lower surface of the filter per high power field ($400\times$) is shown. Cell invasiveness of AdCMV.p53-treated SK-MEL-110 cells and SK-MEL-147 cells 1, 3, and 5 d after infection is represented by open squares and solid squares, respectively. In the SK-MEL-110 cell line, a statistically significant difference was seen with respect to pre-treatment values (day 0) and to values from AdCMV.Null-treated cells (open triangles) ($*p < 0.05$). For the SK-MEL-147 cell line the same comparisons did not show significant differences (solid triangles: AdCMV.Null-treated cells). Values are mean \pm SEM of four independent experiments.

after infection with 50 pfu per cell of AdCMV.LacZ (data not shown).

Cell lysates from SK-MEL-110 and SK-MEL-147 cells infected with AdCMV.p53 (10, 20, and 50 pfu per cell) were tested for efficient wt p53 protein expression, which was detected as early as 1 d after infection, as shown in **Fig 1**. Levels of transduced p53 protein increased with vector concentration in both cell lines. No detectable endogenous p53 protein was observed in uninfected or AdCMV.Null infected cells. Longer exposure times of the same Western blot membranes revealed endogenous p53 bands in cell lysates from uninfected and AdCMV.Null-infected SK-MEL-147 cells but not in the corresponding cell lysates from SK-MEL-110 cells (data not shown). The concentration of 50 pfu per cell was therefore chosen to carry out the subsequent experiments.

Effects of wt p53 gene transfer on cell invasion and cell migration To determine whether wt p53 overexpression was associated with modulation of cell invasion, chemoinvasion assays were performed on both cell lines (**Fig 2**). SK-MEL-110 AdCMV.p53-infected cells showed a sustained significant decrease in invasion ability from day 1 after infection ($p < 0.05$ versus pretreatment values). In contrast, the marginal, progressive reduction in invasiveness of AdCMV.Null infected cells did not reach statistical significance at any time and was similar to that of uninfected cells (data not shown). Further, the significant difference in invasion capability between AdCMV.p53- and AdCMV.Null-treated cells, evident from day 1 (16.5 ± 2.9 vs 48.0 ± 8.0 cells per field, $p < 0.05$), persisted at days 3 and 5 after infection (day 3: 11.7 ± 1.4 vs 31.5 ± 4.1 ; day 5: 17.0 ± 4.7 vs 30.0 ± 13.2 ; $p < 0.05$ for both). Interestingly, AdCMV.p53 treatment did not affect SK-MEL-147 cell invasion at days 1, 3, and 5 after infection. These findings show that the p53 overexpression is associated with a strong decrease in the invasiveness of the cells carrying a mutated p53 gene. Cell migration, assessed by chemotaxis assay, of AdCMV.p53- versus AdCMV.Null-treated SK-MEL-110 cells at day 1 (28.3 ± 6.4 vs 45.0 ± 14.5 cells per field, respectively), day 3 (28.3 ± 1.7 vs 42.7 ± 3.0), and day 5 (41.3 ± 5.8 vs 45.7 ± 6.4) showed that Ad-mediated wt p53 gene transfer did not significantly affect cell motility ($p = \text{NS}$ by ANOVA). Further, the effects on cell migration of AdCMV.p53 and AdCMV.Null infection were not significantly different from pretreatment values (48.0 ± 15.3 cells per field at day 0; $n = 3$; $p = \text{NS}$ by ANOVA).

Effects of wt p53 gene transfer on MMPs and TIMP-2 secreted levels As changes in MMP-2 and MMP-9 secretion are often correlated *in vitro* with a modulation of cell invasion, in the absence of change in cell migration, these metalloproteases were quantitated, by direct zymography, in conditioned media from both cell lines. In SK-MEL-110 cells downmodulation of MMP-2,

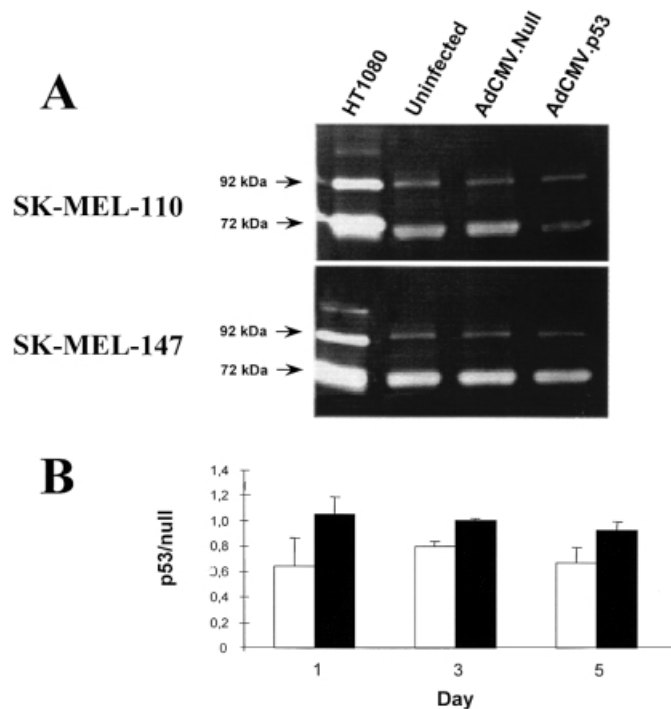


Figure 3. Zymography of conditioned media from SK-MEL-110 and SK-MEL-147 cells. (A) Conditioned media from cells cultured in 0% fetal bovine serum for 24 h are tested to assess MMP secreted levels 5 d after AdCMV.p53 infection. The same amount of secreted protein was loaded per lane (2 μ g). The presence of proteins with gelatinolytic activity is shown by light areas on dark background. Both 72 kDa MMP-2 and 92 kDa MMP-9 are detected. Conditioned media from HT1080 cells are used as positive controls. (B) Bars express the ratio between densities of MMP-2 bands detected in conditioned media from cells infected with AdCMV.p53 *versus* cells infected with AdCMV.Null at all time points. SK-MEL-110 cells are represented by open bars and SK-MEL-147 cells by solid bars. Densities of zymographic bands were measured by densitometric scan. Results are mean \pm SEM of four independent experiments.

evaluated by density quantitation of zymographic bands and expressed in arbitrary units, was found. This effect could be appreciated with respect to values from AdCMV.Null-infected cells in each individual experiment ($n = 4$) at day 1 ($-38 \pm 13\%$), at day 3 ($-20 \pm 4\%$), and at day 5 after treatment ($-3 \pm 12\%$) (Fig 3A, B). Lower levels of secreted MMP-2 were consistently found at all time points in conditioned media collected from AdCMV.p53-treated cells compared with media from AdCMV.Null-infected cells ($p < 0.05$ for the difference in trend by ANOVA). Furthermore, the difference between treatments reached statistical significance at day 5 after infection ($p < 0.01$ by *posthoc* analysis). In contrast, no modulation was observed in SK-MEL-147 conditioned media. Interestingly, in preliminary experiments a decrease in MMP-2 secreted levels after AdCMV.p53 gene transfer was found in conditioned media from a uveal melanoma cell line (OMM-1), characterized in our laboratory for p53 mutations. In contrast, the uveal melanoma cell line SP-6.5, expressing wt p53, did not show MMP-2 modulation after wt p53 gene transfer (manuscript in preparation). These findings suggest that the decrease in secreted MMP-2 after wt p53 overexpression is not unique to the SK-MEL-110 cell line. MMP-9 levels were not affected by wt p53 gene transfer. As TIMP-2 is the main inhibitor of MMP-2, we performed Western blot analysis on conditioned media and neither vector affected TIMP-2 secreted levels (Fig 4).

Effects of wt p53 gene transfer on MMP-2 mRNA levels
The decrease in MMP-2 levels in conditioned media of AdCMV.p53-infected SK-MEL-110 cells could be due to a modulation of MMP-2 gene expression by wt p53. Therefore,

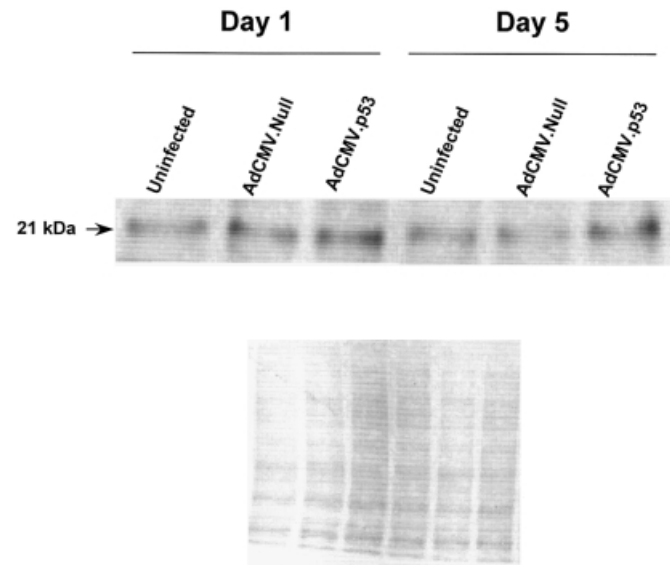


Figure 4. TIMP-2 protein detection in conditioned media from SK-MEL-110 cells. Western blot analysis was carried out on conditioned media collected at days 1 and 5 after wt p53 gene transfer. The same amount of total protein from cell lysates was loaded per lane (10 μ g). Red-Ponceau-stained membrane is shown in the lower panel as a loading control. Secreted levels of TIMP-2 are not modulated by treatment. Similar results were obtained in two separate experiments.

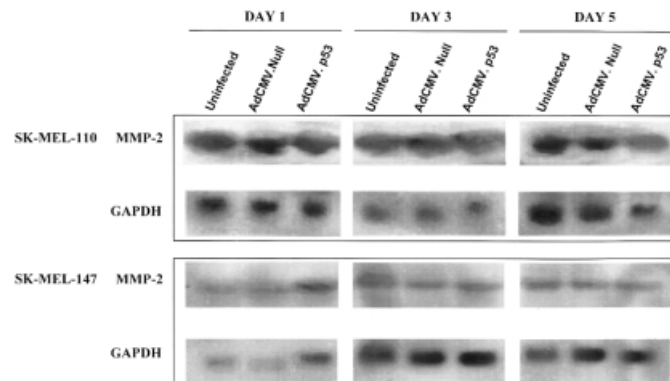


Figure 5. MMP-2 mRNA levels 1, 3, and 5 d after wt p53 gene transfer. Twenty-five micrograms of total RNAs were subjected to northern blot hybridization with a 32 P-labeled cDNA probe for human MMP-2 and visualized by autoradiography. The filters were rehybridized with a GAPDH probe as internal control for RNA loading.

we determined MMP-2 mRNA levels in both cell lines at days 1, 3, and 5 after treatment. After normalizing MMP-2 mRNA by GAPDH mRNA band densities, no effect of Ad-mediated wt p53 gene transfer could be appreciated up to day 5 after infection in either cell line (Fig 5).

Effects of wt p53 gene transfer on net cell proliferation
The inability of p53 overexpression to affect SK-MEL-147 cell invasion and MMP-2 levels could express a failure to respond to AdCMV.p53 treatment. For this reason we evaluated whether net cell proliferation, which accounts for both altered proliferation and death rate, was affected by Ad-mediated wt p53 overexpression (Fig 6A). Both SK-MEL-110 and SK-MEL-147 net cell proliferations decreased after infection with AdCMV.p53. Compared to AdCMV.Null-treated cells, however, SK-MEL-110 net cell proliferation was significantly depressed from day 1, whereas in SK-MEL-147 cells this inhibition became significant only from day 3.

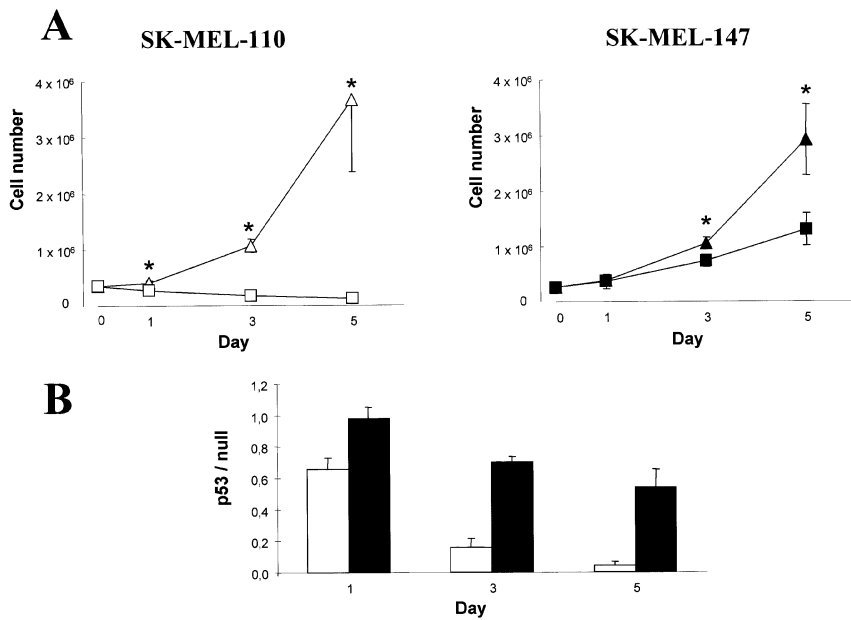


Figure 6. Effect of wt p53 overexpression on net cell proliferation. Cell number was assessed by direct cell counts after the Trypan blue dye exclusion test. Results are expressed as mean \pm SEM of four independent experiments. (A) For both cell lines, cell number is reported after treatment with either AdCMV.p53 (open squares and solid squares) or AdCMV.Null (open triangles and solid triangles). (*: $p < 0.05$ versus AdCMV.p53-treated cells at the same time point). (B) Bars represent the ratio between cell numbers of AdCMV.p53-treated groups and AdCMV.Null-treated groups at all time points. Open bars refer to SK-MEL-110 cells and solid bars to SK-MEL-147 cells.

A direct comparison between the net effects of AdCMV.p53 versus AdCMV.Null treatment in each cell line revealed that growth inhibition was present in SK-MEL-147 cells ($2 \pm 7\%$, $30 \pm 3\%$, and $46 \pm 12\%$) although significantly less pronounced than in SK-MEL-110 ($34 \pm 7\%$, $84 \pm 6\%$, and $96 \pm 2\%$) (Fig 6B).

DISCUSSION

In previous studies, we have demonstrated that Ad-mediated wt p53 gene transfer inhibits melanoma cell growth and induces apoptosis *in vitro* and *in vivo*, significantly decreasing tumorigenicity in nude mice (Cirielli *et al*, 1995). To investigate whether the inability of melanoma cells to grow and metastasize *in vivo* could also be due to additional effects of wt p53 overexpression other than apoptosis, we focused our study on tumor cell invasiveness and on type IV collagenase MMP-2 and MMP-9 modulation in response to Ad-mediated wt p53 gene transfer. In melanoma cells, was observed an increase in the expression of MMP-2 and MMP-9 together with an increase in invasiveness (Hendrix *et al*, 1992). Moreover, it is known that the degradation of the epithelial basal membrane is a prerequisite for the invasion of epithelial melanocytic tumor cells into the dermis and for their consequent systemic spreading. In this study we demonstrated that wt p53 gene transfer decreases *in vitro* cell invasion in a human melanoma cell line, SK-MEL-110, which carries a mutated p53 gene. This effect is associated with a reduction of secreted MMP-2 in conditioned medium. To our knowledge, this is the first time that wt p53 overexpression has been shown to be directly correlated with both an inhibition of *in vitro* tumor cell invasion and a decrease in secreted MMP-2 levels in p53-mutated cells.

The reduction in SK-MEL-110 cell invasiveness occurred concomitantly with enhanced p53 protein levels as quickly as 24 h after Ad-mediated wt p53 gene transfer. Moreover, at baseline, SK-MEL-110 cells exhibited an invasiveness 2-fold higher than the wt p53 SK-MEL-147 cells, suggesting that in the p53-mutated SK-MEL-110 cell line wt p53 restoration negatively modulates such a highly invasive phenotype *in vitro*, reducing it to the same levels expressed by wt p53 tumoral cells (i.e., SK-MEL-147). These results are in agreement with recent findings showing that the functional inactivation of wt p53 gene in lung carcinoma cells by p53-antisense cDNA transfection generated highly invasive clones (Mukhopadhyay and Roth, 1996). In parallel with the observed inhibition of cell invasiveness, a negative modulation of MMP-2 secreted levels was observed in conditioned medium from SK-

MEL-110 cells. These results are consistent with the well-known role of the 72 kDa gelatinase MMP-2 as the predominant basal membrane degrading type IV collagenase in human melanoma (Ray and Stetler-Stevenson, 1995).

Recently, it has been reported that degradation of basement membrane in superficial urothelial carcinomas together with reduction, or total loss, of type IV collagen are significantly related to wt p53 inactivation and/or to overexpression of its feedback negative regulator mdm2 (Ozdemir *et al*, 1997).

It is noteworthy that SK-MEL-110 cell migration, assessed by the chemotaxis assay, was not affected by wt p53 overexpression. This can be explained by the fact that in the chemotaxis assay the concentration of the gelatin used to coat the porous filters was too low to represent a barrier for the cells. Thus, in these experiments the migration of the cells is only representative of cell motility, which did not appear to be modulated by Ad-mediated wt p53 overexpression. A similar result has been previously reported after replacement of the retinoblastoma (RB) tumor suppressor gene in RB-defective tumor cells, whose inhibition of invasiveness, correlated with suppression of tumorigenicity *in vivo*, was not associated with *in vitro* effects on chemotaxis (Li *et al*, 1996).

In contrast with results obtained studying p53-mutated SK-MEL-110 cells, the SK-MEL-147 cell line expressing wt p53 has shown no changes in either cell invasion or secreted MMP-2 levels after wt p53 overexpression.

A significant negative effect of wt p53 gene transfer on net cell proliferation, expressed by the number of viable cells independently of their cell cycle status, was evident in both cell lines, although it was less pronounced in SK-MEL-147 than in SK-MEL-110 cells. This wt p53 inhibitory action has been related to two main mechanisms: the induction of apoptosis (Lane, 1992; Cirielli *et al*, 1995; Yang *et al*, 1995) and/or growth arrest (Gomez-Manzano *et al*, 1997). The effect of AdCMV.p53 on SK-MEL-147 cell numbers rules out the possibility that the lack of effect on cell invasion and MMP-2 levels observed is due to intrinsic insensitivity of this cell line to wt p53 overexpression.

In this study, a modulation of both cell invasion and MMP-2 levels in response to wt p53 overexpression was observed in p53-mutated SK-MEL-110 but not in SK-MEL-147 cells expressing endogenous wt p53. On the other hand, it is not surprising that, in a cell line already expressing a wt p53 protein, no further modulation of some wt-p53-regulated pathways can be achieved by introduction of additional copies of the wt gene.

Further, it must be considered that the loss of p53 tumor suppressor functions may be followed by an alteration of the repertoire of genes controlled by mutant p53 (Lane and Benchimol, 1990; Li *et al*, 1998). Thus, cells from the same tumoral type could respond differently to wt p53 gene transfer as a function of their p53 status (Blagoskolonny, 1997).

A recent report has described an enhancer region in the promoter of the MMP-2 gene that binds to wt p53 protein (Bian and Sun, 1997). Nevertheless, as the induction of MMP-2 mRNA expression did not occur in a p53-dose-dependent manner, Bian and Sun hypothesized that activation of the MMP-2 promoter region is under a more complicated regulation. In this study no evident effect on MMP-2 mRNA expression in SK-MEL-110 cells was detected upon wt p53 gene transfer, despite the decreased MMP-2 secreted levels and the reduced cell invasion observed. As p53, as a multiple activator or repressor gene, could also induce upregulation or downregulation of other genes (Kastan *et al*, 1992; Barak *et al*, 1993; El-Deiry *et al*, 1993; Dameron *et al*, 1994; Miyashita and Reed, 1995; Wang *et al*, 1997), the p53-related MMP-2 modulation may be the result of cross-interactions among multiple factors via a p53-indirect pathway.

The finding that the specific TIMP-2 is not modulated by wt p53 gene transfer suggests that this factor did not play a significant role in the inhibition of SK-MEL-110 cell invasion after wt p53 overexpression.

In this study no modulation of MMP-9 secreted levels following wt p53 replacement in SK-MEL-110 cells was observed, suggesting that this proteolytic enzyme does not play an important role in this regulatory pathway.

In conclusion, for the first time our results provide evidence of a novel regulatory mechanism by which, in a p53-mutated melanoma cell line, wt p53 gene transfer significantly reduces cell invasiveness *in vitro* via a decrease in the secreted levels of MMP-2.

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