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RESEARCH ARTICLE

Exogenous wt-p53 protein is active in transformed cells but not in their non-transformed counterparts: implications for cancer gene therapy without tumor targeting

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Abstract

Background Expression of exogenous wild-type p53 (wt-p53) protein in tumor cells can suppress the transformed phenotype whereas it does not apparently induce detrimental effects in non-transformed cells. This observation may provide a molecular basis for p53-mediated gene therapy of p53-sensitive cancers without the need for tumor targeting.

Methods To understand the molecular mechanisms responsible for this different behavior in tumor versus normal cells, biochemical and functional analyses of exogenous wt-p53 protein were performed on non-transformed C_2C_{12} myoblasts and their transformed counterparts, the C_2-ras cells.

Results The exogenous wt-p53 protein, which induced persistent growth arrest only in transformed C_2-ras cells, was shown to be significantly more stable in transformed than in non-transformed cells. This different stability was due to different p53 proteolytic degradation. Moreover, constitutively, exogenous wt-p53 protein was found to be transcriptionally active only in C_2-ras cells but it could also be activated in C_2C_{12} cells by genotoxic damage.

Conclusions Non-transformed C_2C_{12} cells present regulatory system(s) which control the expression and the activity of exogenously expressed wt-p53 protein probably through degradation and maintenance in a latent form. This regulatory system is lost/inactivated upon transformation. Copyright © 2000 John Wiley & Sons, Ltd.

Keywords proteolytic degradation; transacting activity; tumor targeting; recombinant adenovirus

Introduction

One of the main obstacles in tumor gene therapy with tumor suppressor agents (e.g., toxins, pro-drugs, tumor suppressor genes) is the targeting of cancer cells. Several strategies have been proposed to overcome this problem, including methods for tissue-specific transduction or tissue-specific expression [1,2]. An alternative approach to these strategies might be offered by the *TP53* tumor suppressor gene whose expression seems to be detrimental for tumor cells while it is not harmful to normal ones. In early studies demonstrating the tumor suppressor function of the *TP53* gene, rather than its oncogenic properties, it was observed that expression of exogenous wt-p53

protein in normal cells, such as rat embryo fibroblasts [3,4], or in non-malignant cells, such as colon adenoma cells [5], caused no detectable phenotypic changes. More recently, it was shown that expression of exogenous wt-p53 causes no observable changes in normal human bronchial epithelial cells [6], and primary bone marrow cells [7]. These results were also confirmed *in vivo* by adenovirus-mediated expression of exogenous wt-p53 in the lung [6]. We previously observed that non-transformed 32D myeloid progenitors can be forced to stably express an exogenous wt-p53 protein without affecting morphology, viability, proliferation, and differentiation capacity [8]. However, when these wt-p53-expressing 32D cells were transformed by different activated oncogenes, they became sensitive to wt-p53 suppressing activity and directly responded to exogenous wt-p53 expression with growth arrest or differentiation [9]. These observations suggest that in non-transformed cells the apparatus necessary to control the activity of the endogenous p53 protein, activation of the latent form and inactivation/degradation of the active form, can also control the exogenous protein. In contrast, tumor cells might lack this type of regulation and, therefore, exogenous wt-p53 protein is active and can suppress the transformed phenotype. On the basis of these observations, we have recently proposed that bone marrow purging of leukemia cells might be achieved *ex vivo* by transduction of the *TP53* gene in all marrow cells, normal and leukemic, with the functional inactivation only of the latter ones [7–10]. This gene therapy approach would overcome the requirement for tumor targeting.

Here, we investigate the mechanisms responsible for the divergent responses of normal and tumor cells to exogenous wt-p53 expression. For this purpose, we developed an experimental model based on a single cell type, the non-transformed C_2C_{12} murine myoblasts, and their transformed counterparts, the C_2-ras cells, obtained by expression of the v-*Ha-ras* oncogene. As observed in other reports, exogenous wt-p53 expression exerted strong suppressing activity only in v-*Ha-ras*-transformed cells. To evaluate the basis of this different behavior, expression and transcriptional activity of exogenous p53 were analyzed. We found that exogenous wt-p53 protein had a shorter half-life in C_2C_{12} than in the transformed counterparts due to different proteolytic degradation. Comparable results were obtained in normal and human-derived cells. Moreover, the p53 protein was transcriptionally active only in transformed C_2-ras cells. However, the absence of exogenous wt-p53 activity in non-transformed C_2C_{12} cells was not constitutive since it could be activated by genotoxic damage. Taken together, these results show that the precise and multiple mechanisms of p53-regulation present in normal cells are able, at least within a certain range, to control expression and activity of an exogenous wt-p53 protein. These findings may explain the tolerance of normal cells to exogenous wt-p53 transduction and strongly support the possibility of *TP53*-mediated gene therapy without the need for tumor targeting.

Materials and methods

Cells and culture conditions

C_2C_{12} murine myoblasts [11], C_2-ras, C_2-myc [12], C_2-src transformed cells [13] (kindly provided by M.C. Gauzzi, University of L'Aquila, Italy), LI human glioblastoma cells [14], WI38, and MC5 human normal fibroblasts (kindly provided by S. Bacchetti, Mc Master University, Ontario, Canada) were cultured in DMEM (GIBCO-BRL, Life Technology, Grand Island, NY, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS; GIBCO-BRL), and penicillin/streptomycin (GIBCO-BRL). C_2-ras transformed cells were obtained either by infection with the replication-defective ras-zip recombinant ecotropic retrovirus, coding for v-*Ha-ras* oncogene and neomycin resistance gene [15], or by infection with the replication-competent, v-*Ha-ras* expressing murine sarcoma virus (MSV). Ras-zip-expressing cells were selected in the presence of G418 and cloned (clone #9 was chosen for the experiments because differentiation capacity was completely abolished). C_2-ras-zip#9 cells were used for *in vivo* experiments to avoid retroviral infection of mice. MSV-expressing cells were maintained as a polyclonal population and were used for *in vitro* experiments to avoid clone-dependent artefacts. Both v-*Ha-ras*-carrying retroviruses were kindly provided by F. Tatò ('La Sapienza' University, Rome, Italy).

Tumorigenicity

The tumor-inducing capacity of C_2C_{12} and C_2-ras cells was determined by injecting 1×10^3, 1×10^4, or 1×10^5 cells subcutaneously, between the scapulae, in syngeneic C3H/HeJ adult mice. Three mice were injected for each cell group. The appearance of palpable tumors was assessed weekly for 6 months.

Recombinant adenoviruses and adenoviral infection

The following adenoviruses were used: the recombinant adenovirus Ad-wt-p53 and Ad-mutp53, which carries the human *TP53* or the human $p53Ser^{135}$ mutant cDNAs, respectively, under the control of the CMV promoter [16], the recombinant adenovirus AdCMV-LacZ, which carries the β-galactosidase gene under the control of CMV promoter [16]. These viruses were kindly provided by S. Bacchetti, and F. Graham (McMaster University, Hamilton, Ontario, Canada). The deletion-mutants dl520, which lacks E1A 13S, and the dl312, which lacks the entire E1A region were kindly provided by A. M. Lewis Jr. (FDA, Bethesda, MD, USA) [17]. All these adenoviruses are defective for replication.

Virus stocks were amplified on a large scale in 293 cells

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and viral titers were determined as plaque forming units/ml (pfu/ml) by a plaque test assay as previously described [18]. Adenoviral infections were carried out on cell monolayers, at the indicated multiplicity of infections (MOIs), by 1 h incubation at 37°C in the presence of a thin layer of medium. Fresh culture medium was then added for the indicated periods of time. To verify whether the Ad-wt-p53 behaved as reported by others, we infected the human rhabdomyosarcoma cell line RD to test the effect of Ad-wt-p53 on proliferation [19].

Proliferation curves and cell cycle analysis

Exponentially proliferating cells were infected by Ad-wt-p53, or dl312 at different MOIs; medium alone was used for mock infection. Twenty-four hours post-infection 4×10^4 cells were seeded on 60 mm Petri dishes. Cell numbers were determined in duplicate, at daily intervals, by direct counting with a hemocytometer. Cell viability was determined by trypan blue exclusion. Numbers are means of three different experiments.

For cell cycle analysis cells were infected as above and, 48 h later, incubated in the presence of 10 μM BrdU (Sigma, St. Louis, MO, USA) for 3 h, washed with cold PBS, fixed, and stained with anti-BrdU moAb (DAKO, A/S, Denmark) as previously described [20]. DNA was counter-stained with PI (Sigma, St. Louis, MO, USA). FITC-positivity and DNA content were analyzed by an Epics XL analyzer (Coulter Corporation, Miami, FL, USA).

β-galactosidase assay

Adenoviral infections with AdCMV-LacZ were carried out on cell monolayers at 100 pfu/cell, by 1 h incubation at 37°C as described above. Forty-eight, 72, and 96 h after infection cells were collected in 0.25 M Tris-HCl-pH 7.8 and lysed by three cycles of freezing and thawing. For *β*-gal assay, cell lysates containing equal amounts of protein were incubated at 37°C with 2 mg/ml ONPG (*o*-nitrophenyl *β*-D-galactopyranoside) (Sigma), 10 mM KCl, 1 mM $MgCl_2$, 50 mM *β*-mercaptoethanol until reactions turned yellow. Optical density (OD) values were subsequently obtained at a wavelength of 420 nm. Numbers are means of three independent experiments.

Metabolic labeling and immunoprecipitation

Cells were infected with Ad-wt-p53 in 25 cm^2 flasks. After 48 h the cells were labeled in the presence of 100 μCi/ml of [^{35}S]-methionine (Dupont/New England Nuclear Italian s.p.a. Milano, Italy), and chased as previously described [13]. For immunoprecipitation analysis, cell lysates containing equal amounts of proteins were incubated with anti-p53 PAb1801 (Ab2, Oncogene Science, Uniondale, NY, USA). Immunocomplexes were analyzed on 10% SDS-PAGE, and subjected to fluorography. Gels scan was performed on a ScanJet IIc (Hewlett Packard) and analyzed by a Molecular Dynamics, Image Quant.

Western blot analysis

Total cell lysates were prepared and equal amounts of proteins were electrophoresed on SDS-and blotted onto nitrocellulose membranes (Bio-Rad, Hercules, CA, USA). Filters were reacted with sheep anti-p53 polyclonal antibody (Ab7 Oncogene Science), or anti-E1A M73 moAb (kindly provided by A. Giordano, Jefferson University, Philadelphia, PA, USA) as previously described [7]. Immunoreactivity was detected by the ECL chemo-luminescence reaction kit (Amersham Corp., Arlington Heights, IL, USA), following the manufacturer's instructions.

Chloramphenicol acetyltransferase CAT assay analysis

PG-cat#6 cells [20] are C_2C_{12} cells stably transfected with the PG_{13}CAT vector, carrying the CAT reporter gene driven by polyoma virus minimal promoter and 13 copies of the p53-consensus sequence [21], kindly provided by B. Vogelstein, (Johns Hopkins Oncology Centre, Baltimore, MD, USA). These cells were transformed by MSV infection (PG-cat#6-ras) as reported above. C_2C_{12} cells, stably transfected with the p(mdm2)NA-CAT vector, which carries the p53-dependent intronic promoter of the murine *mdm2* gene [29] and subsequently transformed by MSV infection, are referred to as mdm2-cat and mdm2-cat.ras, respectively. CAT activity was determined using the two-phase fluor diffusion assay as described [22]; counts per minute (cpm) were normalized to protein quantity. For wt-p53 activation, cells were treated with 0.5 μg/ml doxorubicin (Adriamycin-ADR) for 7 h before CAT assay. Data shown are representative results of three independent experiments.

Results

Transformation of murine C_2C_{12} myoblasts by the v-*Ha-ras* oncogene

To study the mechanisms involved in the different response to exogenous wt-p53 expression of non-transformed versus transformed cells, we developed an experimental model based on a single cell type. We used the non-transformed C_2C_{12} murine myoblasts and their transformed counterparts, the C_2-ras cells, obtained by expression of the v-*Ha-ras* oncogene. C_2C_{12} cells are immortal myoblasts [11] with endogenous *TP53* gene [20]. We used either the replication-defective zip-ras [15], or the replication-competent MSV retroviruses to infect C_2C_{12} cells (for details, see Materials and methods). As expected, v-*Ha-ras* expression suppressed differentiation (data not shown) and induced *in vivo* tumorigenicity as assessed by subcutaneous injection of serial dilutions

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Table 1. *In vivo* tumorigenesis of C_2-ras cells

Cell lines	1×10^{3a}	1×10^4	1×10^5
C_2C_{12}	0/3[b]	0/3	0/3
C_2-ras-zip#9	2/3	2/3	3/3

[a]Number of cells injected in mice.
[b]Number of mice with tumour/number of total mice injected.

of C_2-ras-zip#9 and parental C_2C_{12} cells into C3H/HeJ syngeneic mice (Table 1). These results indicate that C_2C_{12} myoblasts underwent full tumor transformation upon v-*Ha-ras* transduction.

Infection with wt-p53-recombinant adenovirus induces strong functional effects only in C_2-ras transformed cells

To evaluate the response to the exogenous *TP53* gene transduction, parental C_2C_{12} and C_2-ras transformed cells were infected at two different MOIs, 20 and 90 pfu/cell, with a recombinant adenovirus carrying the human *TP53* cDNA under the control of the CMV promoter (Ad-wt-p53) [16]. Ad-wt-p53 infection significantly inhibited proliferation of C_2-ras cells at both MOIs, whereas it slightly reduced C_2C_{12} proliferation only at the highest MOI (Figure 1A). Inhibition of C_2-ras proliferation was due to cell accumulation in both G_1 and G_2/M phases of the cell cycle (see below). Viability was not significantly affected in either cell type at these MOIs (Figure 1B). However, at higher MOIs (200 and 300) C_2-ras cells underwent apoptosis while C_2C_{12} cells were only temporarily slowed down in their cell cycle and recovered their normal rate of proliferation within 48 h (data not shown). These results show that C_2-ras transformed cells are much more sensitive to exogenous wt-p53 suppressing activity than parental C_2C_{12} cells. Thus, these cells are a suitable model for studying the different responses of non-transformed and transformed cells to exogenous wt-p53 expression.

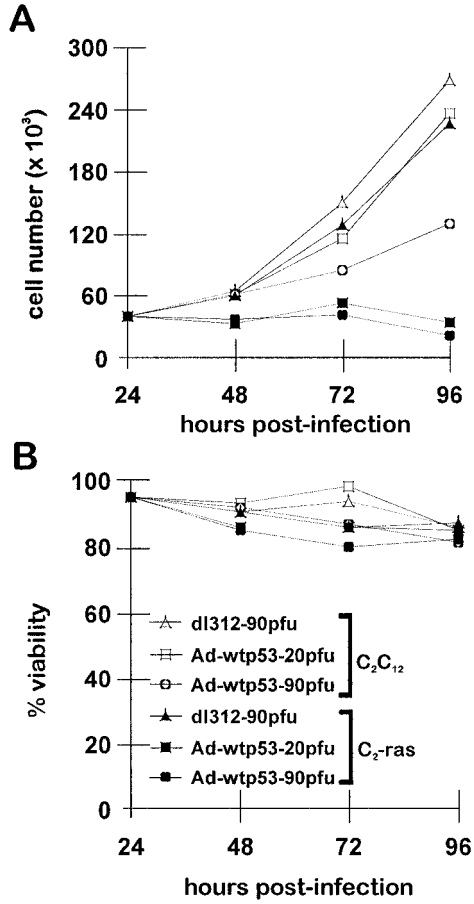

Figure 1. Analyses of proliferation rate and cell viability after Ad-wt-p53 and dl312 infection. (A) C_2C_{12} myoblasts and C_2-ras cells were infected at MOIs 20 and 90 with Ad-wt-p53 or at MOI 90 with dl312. Twenty-four hours after infection, 4×10^4 cells were plated in 60 mm dishes and harvested at the indicated times. Cell numbers were determined daily in duplicate. White symbols represent C_2C_{12} myoblasts, black symbols represent C_2-ras cells. (B) Cell viability was determined at daily intervals by the trypan blue exclusion test on the same cells used for the proliferation experiments. Cells infected at MOI 90 with the dl312 adenovirus were used as controls in each experiment (see Materials and methods)

Exogenous wt-p53 protein is more stable in C_2-ras than in C_2C_{12} cells

To verify the expression levels of exogenous p53 protein in the C2-cell model, C_2C_{12} and C_2-ras cells were infected with Ad-wt-p53 at MOIs 20 or 90 and analyzed at different times post-infection. As shown in Figure 2A, the time-course analysis revealed high and persistent p53 expression in C_2-ras cells at both MOIs, with peak expression 72 h post-infection. Conversely, exogenous wt-p53 protein was mostly present in C_2C_{12} cells 48 h post-infection at the highest MOI and dramatically decreased during the following 2 days. Moreover, by infecting with the same MOI of Ad-wt-p53 the expression level of exogenous p53 was higher in C_2-ras than in C_2C_{12} cells (Figure 2A). The differences in protein levels and expression kinetics might be due to different regulation of exogenous wt-p53 protein by the two cell types. Alternatively, these differences might be determined by the experimental procedures and depend on differences in sensitivity to adenovirus infection, different activity of the CMV promoter driving the *TP53* gene, or dilution effects in proliferating cells. To evaluate these possibilities, the cells were first infected at the same MOI (90 pfu/cell) by E1A-expressing dl520 adenovirus (see Materials and methods). As shown by Western blotting, 48 h post-infection C_2C_{12} and C_2-ras cells expressed similar levels of E1A protein (Figure 2B), indicating that these cells can be infected by adenovirus and express a viral protein driven by its own promoter at comparable levels.

To evaluate the second possibility, CMV promoter activity was analyzed by infection with the adenoviral vector AdCMV-LacZ carrying the *β-gal* gene under the control of the CMV promoter [16]. Figure 2C shows that, at a similar MOI – 100 pfu/cell – AdCMV-LacZ induced similar kinetics of β-gal activity in both cell types even though the absolute values were different, being fourfold higher in C_2-ras than C_2C_{12} cells 48 h post-infection,

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suggesting a different CMV promoter activity in the two cell types. To compensate for this different CMV promoter activity, we took advantage of the possibility of obtaining similar levels of exogenous protein expression by infecting C_2-ras and C_2C_{12} cells at different MOIs: 20 and 90 pfu/cell, respectively (Figure 2D). We refer to this type of infection as 'differential infection', and all the following experiments were performed with this type of infection.

To ascertain that infectability was also similar at the single cell level, an indirect immunofluorescence for p53 and β-gal activity were performed after differential infection. A comparable number of positive cells were found in both cell types in both experiments (data not shown).

Taken together, the results indicate that C_2C_{12} and C_2-ras cells can be equally infected by adenovirus but the CMV promoter has fourfold higher activity in the transformed cells, which, however, does not justify the different kinetics of β-gal and p53 expression (compare Figure 2A and 2C). To exclude the third possibility (i.e. the dilution of non integrated viral DNA in proliferating cells), the stability of exogenous wt-p53 protein was evaluated by pulse-chase experiments. Forty-eight hours after Ad-wt-p53 differential infection the cells were metabolically labeled, chased, and harvested at the indicated times. As shown in Figure 3A, exogenous wt-p53 protein was more rapidly degraded in C_2C_{12} than in C_2-ras cells. The half-life of the exogenous wt-p53 was as short as 20 min in C_2C_{12} cells, which is comparable to the physiological half-life of wt-p53 protein [23], whereas it was about 110 min in C_2-ras cells (Figure 3B). To ascertain whether this behavior is general, rather than ras or mouse cell-specific, the pulse-chase experiment was also performed on C_2C_{12} cells transformed by v-myc [12] and v-src [13], on the LI human glioblastoma cell line [14] and on the WI38 and MC5 normal human fibroblasts. The half-lives of wt-p53 increased in the transformed cell lines, being 90 min in C_2-myc, 60 min in C_2-src, and 210 min in LI cells, indicating that stability of the exogenous wt-p53 in not directly dependent on *ras* oncogene. In contrast, the half-lives of wt-p53 in the normal human fibroblasts was 20 min in WI38 and 25 min in MC5.

To verify whether this different protein stability was specific of the p53 protein in wild-type configuration, C_2C_{12} and C_2-ras cells were infected with a recombinant adenovirus carrying the *p53Ser*^{135} mutant gene (Ad-mutp53) [16]. Both cell types were differentially infected with Ad-mutp53. A time-course analysis of protein expression showed a comparable amount of this mutant p53 in the two cell types (Figure 3C).

Taken together, these results show that exogenous wt-p53 protein is maintained in non-transformed cells with a

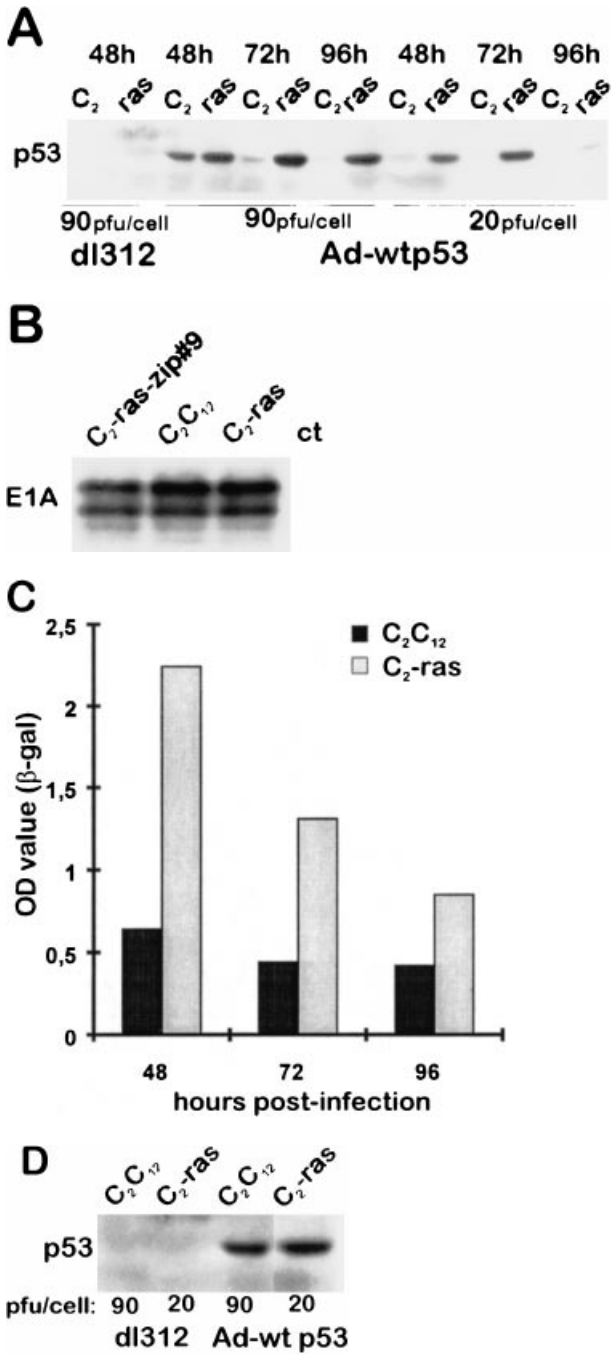

Figure 2. (A) Western blot analysis of p53 protein in C_2C_{12} (C_2) and C_2-ras (ras) cells after infection at MOIs 90 and 20 with Ad-wt-p53. Cells were lysed 48, 72, and 96 h post-infection. Control samples are cells infected at an MOI of 90 with dl312 and lysed 48 h post infection. Equal amounts (20 μg) of proteins were separated on a denaturing 12% SDS-PAGE. Western blot analysis was performed with polyclonal antibody Ab-7. The position of p53 is indicated. (B) Western blot analysis of E1A protein in the indicated cells, after dl520 infection. A similar MOI (90 pfu/cell) of dl520 was used to infect each cell type. Multiple bands of E1A are indicated. The control sample (ct) is represented by C_2-ras cells infected with Ad-wt-p53. (C) Time-course analysis of β-gal activity induced by AdCMV-LacZ infection. C_2C_{12} and C_2-ras cell lysates containing equal amounts of proteins were analyzed 48, 72, and 96 h after AdCMV-LacZ infection at the same MOI (100 pfu/cell). The relative stability of β-gal expression in C_2C_{12} cells at 96 h, compared to 72 h, is probably due to longer persistence of adenoviral genomes determined by the reduction of proliferation induced by cell contact only in these non-transformed cells. (D) Western blot analysis of p53 expression in C_2C_{12} and C_2-ras cells after infection by Ad-wt-p53 at MOIs of 90 and 20, respectively. These differential infections induce expression of comparable levels of exogenous p53 48 h post-infection. Infection with dl312 was used as a negative control

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half-life similar to that of the endogenous wt-p53, while it acquires a longer half-life in the transformed cells, independently of the transforming oncogene and the cell type. In addition, the half-life regulation is specific of exogenous p53 protein in wild-type configuration since an exogenous mutant p53 is comparably stable in both cell types. The latter result is in apparent contradiction to the recent observation that the stability of mutant p53 is not intrinsic to the protein but depends on the tumor cell environment [25]. The different behavior of our mutant p53 could be due either to the fact that we compared non-transformed and transformed cells rather than different transformed cell lines with diverse stability of the endogenous p53 protein. Another explanation might be linked to the type of mutant. We used the p53Ser^{135} which has not been tested for stability in different tumor contexts [25].

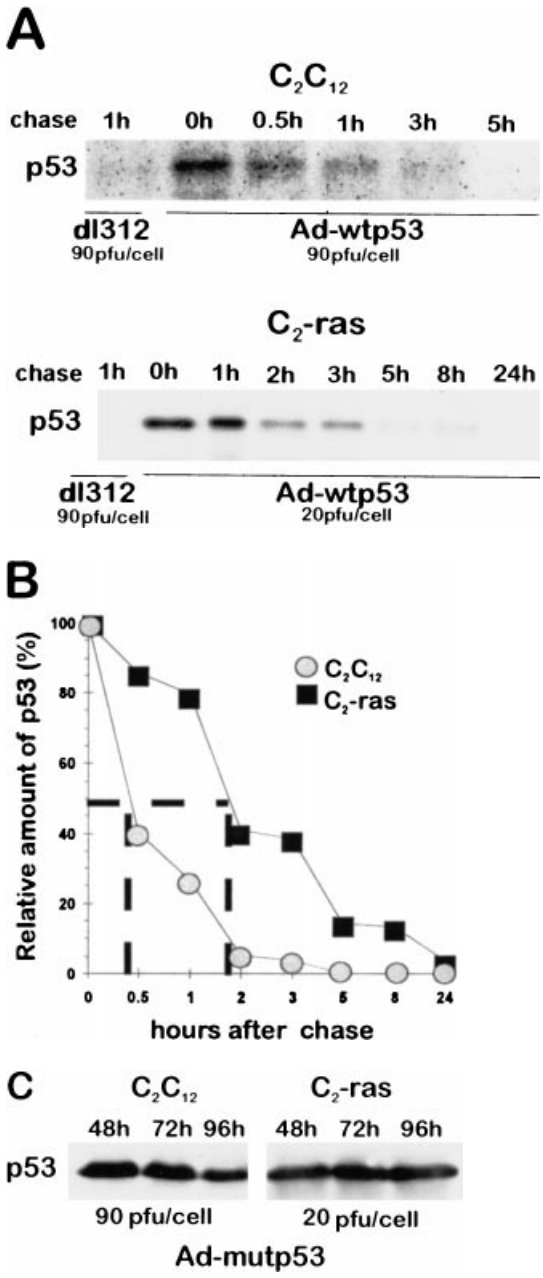

Figure 3. (A) Half-life of exogenous wt-p53 in C_2C_{12} and C_2-ras cells infected with Ad-wt-p53 at MOIs of 90 and 20, respectively. Forty-eight hours post-infection cells were starved for 60 min in methionine-free media and then pulse-labeled for 1 h with [^{35}S]-methionine. The cells were then chased and harvested at the indicated times. Immuneprecipitation of human p53 was performed on cell lysates containing equal amounts of proteins by anti-p53 moAb PAb1801. Cells infected with 90 pfu/cell of dl312 were used as control. (B) p53 levels from panel B were quantitated by densitometry and plotted. The half-life of p53 was about 20 min in C_2C_{12} cells (circle symbols) and about 110 min in C_2-ras cells (square symbols). The half-lives are indicated by dotted lines. (C) Analysis of mutant p53 expression. C_2C_{12} and C_2-ras cells were infected with Ad-mutp53 at MOIs of 90 and 20, respectively, and protein lysates were obtained at the indicated times post-infection

Different proteolytic degradation is responsible for the different stability of p53 in C_2C_{12} and C_2-ras cells

It has been shown recently that the normal turnover of wt-p53 can occur *in vitro* and *in vivo* through ubiquitin-proteasome pathway [26]. To determine whether the different stability of exogenous wt-p53 in C_2C_{12} and C_2-ras cells was due to a different involvement of the proteasome in p53 degradation, we examined p53 levels in C_2C_{12} and C_2-ras cells exposed to the specific proteasome inhibitor MG132 [26]. Both cell types were 'differentially infected' with Ad-wt-p53 and, 48 h post-infection, treated for 4 h with MG132. As shown in Figure 4A, Western blot analysis revealed a substantial increase of p53 steady-state levels in C_2C_{12} cells after proteasome inhibition (25-fold by densitometric analysis); in contrast, p53 level did not change as much in C_2-ras cells (threefold). These data demonstrate that exogenous p53 is degraded through the proteasome pathway much more efficiently in C_2C_{12} than in C_2-ras cells. To determine whether the stabilization of exogenous p53 in transformed versus non-transformed cells is due to a reduced p53 ubiquitination, we performed p53 immunoprecipitation of both cell lysates after proteasome inhibition. A subsequent Western blot analysis revealed a ladder of p53 specific bands ranging from 60 to 90 Kd preferentially in C_2C_{12} cells (Figure 4B). The band size corresponds to species of p53 conjugated with two/five ubiquitin molecules, as previously reported [26]. The ladder of ubiquitin-p53 conjugates was less evident in C_2-ras cells suggesting that tumor transformation might act on exogenous p53 stabilization through either a reduction of p53 ubiquitination or an increase of p53 deubiquitination.

Similar levels of exogenous wt-p53 protein still have suppressing activity only in C_2-ras cells

The experiments described above indicate that C_2C_{12} and C_2-ras cells regulate the stability of exogenous wt-p53 protein in a different fashion. To evaluate whether this mechanism is the only one responsible for the different functional activity of exogenous wt-p53 in non-

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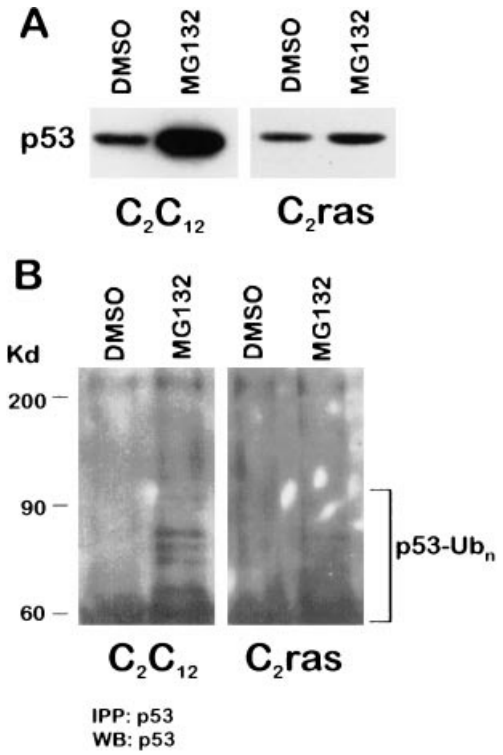

Figure 4. (A) Western blot analysis of p53 expression in C_2C_{12} and C_2ras cells after proteasome inhibition. Cells were either mock-treated (DMSO) or exposed to 0.01 mM concentration of MG132 proteasome inhibitor. Cell extracts were resolved on 9% SDS-and p53 levels were determined by Western blotting using the Ab7 antibody. (B) Cells were treated as in A, lysed, and subjected to immuneprecipitation with moAb1801. Western blot analysis with Ab7 was performed to detect ubiquitin-p53 forms as a ladder between 60 and 90 Kd

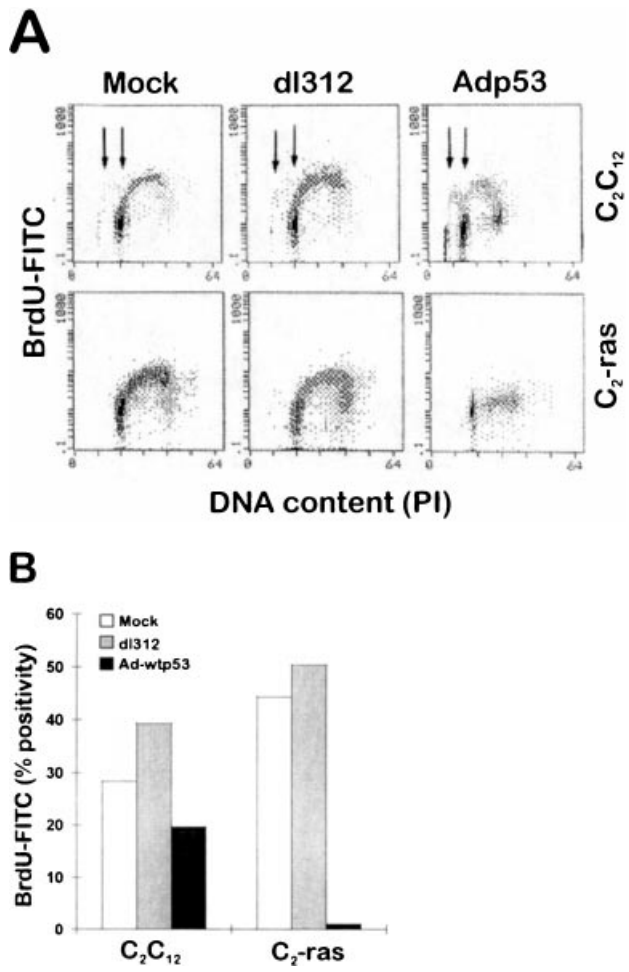

Figure 5. (A) Cell cycle analyses of C_2C_{12} and C_2-ras cells (Mock-, dl312-, and Ad-wt-p53-infected) measured by BrdU incorporation and DNA-content analyses with flow cytometry. Cells were pulsed for 3 h with 10 μM BrdU 48 h after differential infection and analyzed simultaneously for DNA synthesis, with a FITC-conjugated anti-BrdU antibody (vertical axis), and for DNA content, with propidium iodide (horizontal axis). The arrows indicate the two G_1 subpopulations present in C_2C_{12} cells [41]. The absence of dead cells was evaluated by trypan blue exclusion just before fixation and immunostaining (not shown). (B) Quantitation of the S phase as a percentage of BrdU-FITC positivity analyzed in panel A

transformed versus transformed cells, we verified that similar levels of exogenous wt-p53 expression, obtained 48 h post differential infection (Figure 2D), are still able to induce a different response in C_2C_{12} cells versus C_2-ras cells. For this purpose, cell cycle analyses were performed by bromo-deoxyuridine (BrdU) incorporation and propidium iodide (PI) staining. Exogenous wt-p53 only mildly reduced the percentage of C_2C_{12} cells in the S phase of the cell cycle, whereas it induced a strong inhibition of DNA synthesis and an accumulation in the G_1 and G_2/M phases in C_2-ras cells (Figure 5A and 5B). These results show that exogenous wt-p53, even when expressed at similar levels in C_2C_{12} and C_2-ras cells, is still preferentially active in the latter, fully transformed cells. Moreover, lack of exogenous p53 activity in non-transformed C_2C_{12} cells does not depend only on its expression levels.

Exogenous wt-p53 is transcriptionally active only in C_2-ras cells, but it can be activated in C_2C_{12} cells upon DNA damage

Several studies have confirmed that p53 transcriptional activity is important for its suppressor functions [27,28]. We thus investigated whether the presence of different wt-p53 transcriptional activity between C_2C_{12} and C_2-ras cells might contribute to the different functionality observed in these two cell types. To this end, C_2C_{12} cells stably transduced with the PG_{13}CAT construct, which carries 13 wt-p53 DNA-consensus binding sites upstream to a CAT reporter gene [21] were used. These cells, which we indicate as PG-cat#6 [20], were transformed with Ha-MSV to obtain the PG-cat#6-ras cells (see Materials and methods). The v-*Ha-ras*-induced transformation of PG-cat#6 cells was confirmed by determining the inhibition of muscle differentiation as described above (data not shown). Subsequently, PG-cat#6 and PG-cat#6-ras cells were infected with Ad-wt-p53 at MOIs of 90 and 20, respectively, and 48 h later processed to assess CAT activity. Similar expression of exogenous p53 was assessed by Western blotting and immunofluorescence (data not shown). Mock- or dl312-infected cells

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were used as controls. As shown in Figure 6A, exogenous wt-p53 protein was transcriptionally active only in PG-cat#6-ras cells. To evaluate whether the exogenous wt-p53 protein is constitutively inactive in non-transformed C_2C_{12} cells, or whether it can be activated by stress conditions able to activate the endogenous p53 protein, DNA damage was induced in these cells by Adriamycin (ADR) treatment 48 h after Ad-wt-p53 infection. In these conditions, wt-p53 transcriptional activity was present in mock- dl312- and Ad-wt-p53-infected C_2C_{12} cells (PG-cat#6) (Figure 6B). However, CAT activity of Ad-wt-p53-infected cells was increased by about 35% over mock-infected cells and 92% over dl312-infected cells, indicating that the exogenous p53 protein can also be activated by ADR-treatment. Interestingly, in the ras-transformed cells the endogenous wt-p53 protein (mock columns in Figure 6A) had twice as much activity on the $PG_{13}CAT$ construct as that observed in C_2C_{12} cells, suggesting that the endogenous protein is also, at least partially, in a transcriptionally active form. Similar results were obtained with $PG_{13}CAT$-transduced C_2C_{12} cells maintained as a polyclonal population and with their MSV-transformed counterparts (data not shown).

Since the $PG_{13}CAT$ vector carries an artificial promoter, we asked whether the differences in p53 transcriptional activity between C_2C_{12} and C_2-ras cells were also present in a more 'physiological' context. To address this question, C_2C_{12} cells were stably transfected with the p(mdm2)NA - CAT vector, which carries the p53-dependent intronic promoter of the *mdm2* gene [29]. These cells were maintained as a polyclonal population and subsequently transformed by MSV infection. As shown in Figure 6C, Ad-wt-p53 infection induced CAT activity only in the ras-transformed cells (compare column 5 with 11). However, in parental C_2C_{12} cells, ADR treatment was able to activate both endogenous and exogenous p53 proteins as measured on the $PG_{13}CAT$ vector (compare column 1 with 2, and 5 with 6). Also consistent with the experiments performed with the $PG_{13}CAT$ vector, the endogenous p53 protein of ras-transformed cells was spontaneously transcriptionally active, the CAT activity being 16-fold higher than in parental C_2C_{12} cells (compare columns 1 and 7). Nevertheless, ADR treatment of ras-transformed cells was able to increase the CAT activity mediated by endogenous and exogenous wt-p53 proteins (compare columns 7 with 8 and 11 with 12).

To further confirm the transcriptional activity of exogenous wt-p53 protein in our C2-cell model, the expression of endogenous mdm2 mRNA was analysed by Northern blot assay. Consistent with the results obtained with the *mdm2* promoter, in the CAT assay, exogenous p53 expression increased the mdm2 mRNA levels in C_2-ras cells but not in C_2C_{12} cells (Figure 7A and 7B).

Taken together, these results show that the different responses to exogenous wt-p53 expression in non-transformed and transformed cells also depend on differences in transcriptional activity. Moreover, they suggest that the exogenous wt-p53 protein, as well as the endogenous one, is maintained in a latent form in

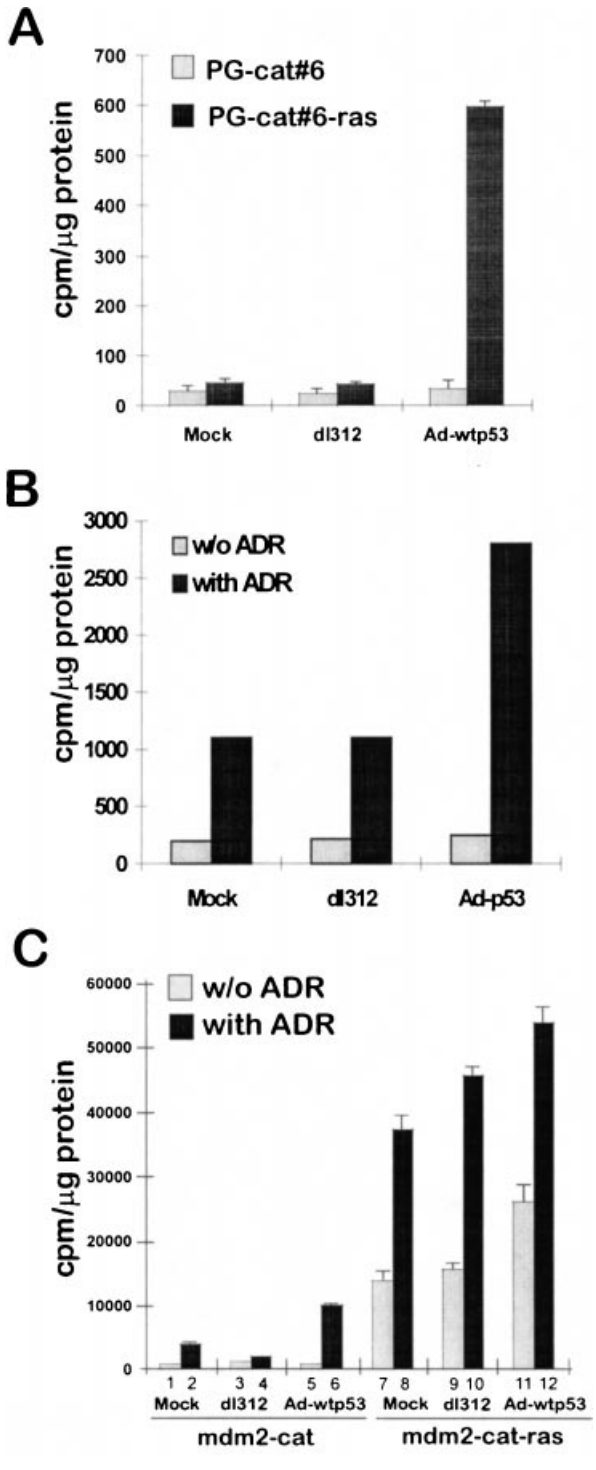

Figure 6. (A) Induction of CAT activity by exogenous wt-p53 in stably transfected PG-cat#6 and PG-cat#6-ras cells. Cells were tested for CAT activity 48 h after infection with Ad-wt-p53 (90 and 20 pfu/cell, respectively) or dl312 (90 pfu/cell). Means and standard deviations from three different experiments are indicated. (B) Induction of CAT activity by exogenous wt-p53 after genotoxic damage in PG-cat#6 cells. Cells were infected with 100 pfu/cell of Ad-wt-p53 and 48 h post-infection treated with ADR for 7 h, then harvested for CAT analyses. The data presented are representative results of three independent experiments. (C) Induction of CAT activity by exogenous wt-p53 in stably transfected C2-mdm2-cat (mdm2-cat) and C2-mdm2-cat-ras (mdm2-cat-ras) cells with and without ADR treatment. Experimental conditions are as in panel A

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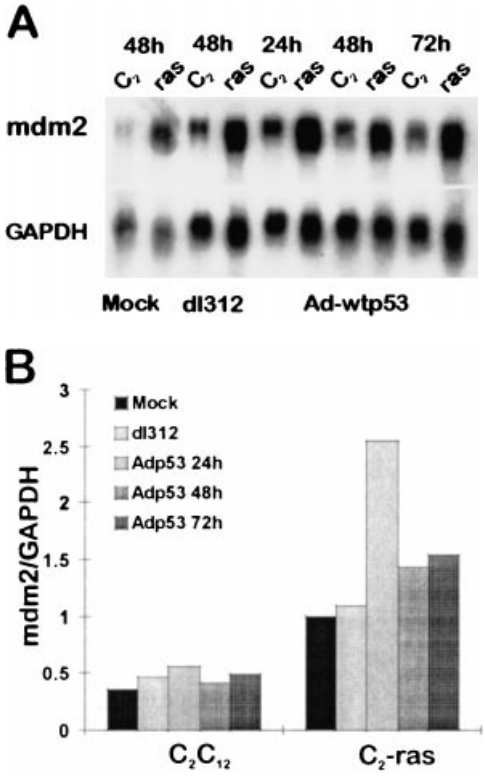

Figure 7. (A) Induction of mdm2 transcripts in C_2C_{12} (C_2) and C_2-ras (ras) cells after p53 expression 48 h post differential infection. Mock-, dl312- and Ad-wt-p53-infected cells are reported. GAPDH expression is used to normalize RNA loading. (B) mdm2 and GAPDH mRNA expression from panel A were quantitated by densitometry and plotted

non-transformed C_2C_{12} cells, as long as its function is not requested. However, it can be activated by stress conditions. In contrast, transformed C_2-ras cells are no longer able to keep wt-p53 protein in an inactive state, and it can be further activated by ADR-induced DNA damage.

Discussion

Several reports have indicated that forced expression of exogenous wt-p53 protein has suppressing activity in tumor cells, while it does not apparently induce detrimental effects in non-fully transformed cells [3–8]. These observations have provided a rationale for the development of tumor gene therapy approaches with the *TP53* gene without the need to target tumor cells [7,10], which is one of the most critical steps in tumor gene therapy with suppressing agents [1,2]. Therefore, the study of the molecular mechanisms responsible for the divergent activities of exogenous wt-p53 protein expression between transformed and non-transformed cells can provide useful insights for practical as well as biological issues. To minimize the differences between the cellular environments, we developed a model based on non-transformed C_2C_{12} murine myoblasts and their transformed counterparts obtained by v-*Ha-ras* oncogene expression. In keeping with other studies, infection with wt-p53-recombinant adenovirus induced growth arrest in the transformed C_2-ras cells while it only mildly and transiently reduced the proliferation rate of non-transformed C_2C_{12} cells indicating that tolerance to exogenous wt-p53 expression is significantly different in these two cell types. In this report, we demonstrate that these divergent effects are due to different expression levels and different transacting activity of the exogenous p53 protein in the two cell types. In particular, we found that the exogenous p53 protein has a half-life similar to wt-p53 – about 20 min – in C_2C_{12} cells, whereas its half-life is strongly increased – about 110 min – in C_2-ras cells. This different protein stability is due to a distinct ubiquitination and proteolytic degradation of p53 in the two cell types. Moreover, it is not related only to v-Ha-ras-mediated transformation or specifically to mouse cells, since in v-myc and v-src transformed C_2C_{12} cells, as well as in LI human glioblastoma cells exogenous wt-p53 protein is stabilized. In contrast, short half-lives were found in normal human fibroblasts. In addition, we found that exogenous wt-p53 protein is transcriptionally active only in transformed C_2-ras cells, as assessed by the activity on the PG_{13} and *mdm2* promoters, and by the increased levels of endogenous mdm2 mRNA.

These results provide direct biochemical evidence that the exogenous wt-p53 protein exerts its tumor suppressor functions mainly in transformed cells. The mechanisms by which exogenous p53 protein is kept inactive in non-transformed cells remains unclear. Recently, several reports have shown how p53 can be regulated through MDM2-p53 protein–protein interaction with subsequent inhibition of p53 transcriptional activity and degradation of p53 protein [30–33]. Moreover, p53 activation by phosphorylation on serines at the amino terminus was shown to alleviate inhibition by MDM2 [34,35] while the $p19^{ARF}$ protein of the Ink4a tumor suppressor locus was found to promote degradation of MDM2 with consequent stabilization of p53 [36,37]. All these data strongly indicate that MDM2 is a potent regulator of p53 after its activation by genotoxic agents and oncogene expression in primary fibroblasts, or its overexpression in transformed cells. However, it has not yet been clearly shown whether MDM2 is also responsible for the short-lived, inactive form of p53 in normal cells, although the early embryonic lethality of the $mdm2^{-/-}$ mice and the rescue of this phenotype by the *TP53* knock-out genotype strongly support this hypothesis [38,39]. On the basis of these observations, it can be hypothesized that in non-transformed C_2C_{12} cells MDM2 might regulate endogenous as well as exogenous p53 levels and activities. We did not observe, after exogenous wt-p53 protein expression in C_2C_{12} cells, any increment of the mdm2 promoter activity, mdm2 mRNA and protein levels (data not shown), confirming similar reports by others [40], and suggesting two different interpretations. MDM2 is not responsible for the inhibition/degradation of the exogenous p53 in non-transformed cells; alternatively, it is not necessary to increase mdm2 transcription for inhibiting/degrading exogenous wt-p53 protein. Although other

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studies will be required to determine the molecule(s) responsible for the exogenous p53 degradation/inactivation in non-transformed cells, this observation is reassuring with regard to the safety of non-neoplastic cells in p53-mediated gene therapy.

More interestingly, from a therapeutic point of view, the p53 activity present in C_2-ras cells could be further increased by ADR treatment indicating that both endogenous and exogenous p53 proteins are not yet fully activated in these cells. This result supports the idea of treating human cancers with combinations of wt-p53 gene therapy and radio/chemotherapy (reviewed in [10]). Furthermore, the absolute level of p53 activation after ADR treatment was 5.4-fold higher in C_2-ras cells than in their non-transformed counterparts suggesting that a p53 gene therapy approach without tumor targeting might leave normal cells unharmed even in combination with radio/chemotherapy.

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References

1. Kasahara N, Dozy AM, Kan YW. Tissue-specific targeting of retroviral vectors through ligand-receptor interactions. *Science* 1994; **266**: 1373–1376.
2. Etienne-Julan M, Roux P, Pourquard P, Carillo S, Jeanteur P, Piechaczyk M. Cell targeting by murine recombinant retroviruses. *Bone Marrow Transplant* 1992; **9**: 139–142.
3. Eliyahu D, Michalovitz D, Eliyahu S, Pinhasi-Kimhi O, Oren M. Wild-type p53 can inhibit oncogene-mediated focus formation. *Proc Natl Acad Sci U S A* 1989; **86**: 8763–8767.
4. Finlay CA, Hinds PW, Levine AJ. The p53 proto-oncogene can act as suppressor of transformation. *Cell* 1989; **57**: 1083–1093.
5. Baker SJ, Markowitz S, Fearon ER, Willson JKV, Vogelstein B. Suppression of human colorectal carcinoma cell growth by wild-type p53. *Science* 1990; **249**: 912–915.
6. Zhang W-W, Alemany R, Wang J, Koch PE, Ordonez NG, Roth JA. Safety evaluation of AdCMV-p53 *in vitro* and *in vivo*. *Hum Gene Ther* 1995; **6**: 155–164.
7. Scardigli R, Bossi G, Blandino G, Crescenzi M, Soddu S, Sacchi A. Expression of exogenous wt-p53 does not affect normal hematopoiesis: implications for bone marrow purging. *Gene Ther* 1997; **4**: 1371–1378.
8. Blandino G, Scardigli R, Rizzo MG, Crescenzi M, Soddu S, Sacchi A. Wild-type p53 modulates apoptosis of normal, IL-3 deprived, hematopoietic cells. *Oncogene* 1995; **10**: 731–737.
9. Soddu S, Blandino G, Scardigli R, Martinelli R, Rizzo MG, Crescenzi M, Sacchi A. wt-p53 induces diverse effects in 32D cells expressing different oncogenes. *Mol Cell Biol* 1996; **16**: 487–495.
10. Soddu S, Sacchi A. p53: Prospects for gene therapy. *Cytokines, Cellular, and Molecular Therapy* 1998; **4**: 177–185.
11. Yaffe D, Saxel O. Serial passaging and differentiation of myogenic cells isolated from dystrophic mouse muscle. *Nature* 1977; **343**: 76–79.
12. Crescenzi M, Crouch DH, Tatò F. Transformation by myc prevents fusion but not biochemical differentiation of C2C12 myoblasts: mechanisms of phenotypic correction in mixed culture with normal cells. *J Cell Biol* 1994; **125**: 1137–1145.
13. Alemà S, and Tatò F. Oncogenes and muscle differentiation: multiple mechanisms of interference. *Semin Cancer Biol* 1994; **5**: 147–156.
14. Del Bufalo D, Biroccio A, Soddu S, Laudonio N, D'Angelo C, Sacchi A, Zupi G. Lonidamine induces apoptosis in drug-resistant cells independently of the p53 gene. *J Clin Invest* 1996; **5**: 1165–1173.
15. Dotto GP, Parada LF, Weinberg RA. Specific growth response of ras-transformed embryo fibroblasts to tumor promoter. *Nature* 1985; **318**: 472–475.
16. Bacchetti S, Graham FL. Inhibition of cell proliferation by an adenovirus vector expressing the human wild type p53 protein. *Int J Cancer* 1993; **3**: 781–788.
17. Jones N, Shenk T. Isolation of adenovirus type 5 host range deletion mutants defective for transformation of rat embryo cells. *Cell* 1979; **17**: 683–689.
18. Graham F, Prevec L. Manipulation of adenovirus. In *Methods of Molecular Biology*, vol. 7, Murray EJ (ed). Clifton NJ: Humana Press, 1991; 109–128.
19. De Giovanni C, Nanni P, Sacchi A, Soddu S, Manni I, D'Orazi G, Bulfone-Paus S, Pohl T, Landuzzi L, Nicoletti G, Frabetti F, Rossi I, Lollini PL. Wild-type p53-mediated down-modulation of interleukin 15 and interleukin 15 receptors in human rhabdomyosarcoma cells. *Br J Cancer* 1998; **78**: 1541–1546.
20. Soddu S, Blandino G, Scardigli R, Coen S, Marchetti A, Rizzo MG, Bossi G, Cimino L, Crescenzi M, Sacchi A. Interference with p53 protein inhibits hematopoietic and muscle differentiation. *J Cell Biol* 1996; **134**: 193–204.
21. Kern SE, Pietenpol JA, Thiagalingam S, Seymour A, Kinzler KW, Vogelstein B. Oncogenic forms of p53 inhibit p53-regulated gene expression. *Science* 1992; **252**: 1708–1711.
22. Huff CA, Yuspa SH, Rosenthal D. Identification of control elements 3′ to the human keratin 1 gene that regulate cell type and differentiation-specific expression. *J Biol Chem* 1993; **268**: 377–384.
23. Gottlieb TM, Oren M. p53 in growth control and neoplasia. *Biochim Biophys Acta* 1996; **1287**: 77–102.
24. Biroccio A, Del Bufalo D, Ricca A, D'Angelo C, D'Orazi G, Sacchi A, Soddu S, Zupi G. Increase of BCNU sensitivity by wt-p53 gene therapy in glioblastoma lines depends on the administration ashedule. *Gene Ther* 1999; **6**: 1064–1072.
25. Midgley CA, Lane DP. p53 protein stability in tumour cells is not determined by mutation but is dependent on Mdm2 binding. *Oncogene* 1997; **15**: 1179–1189.
26. Maki CG, Huibregtse JM, Jones PM. *In vivo* ubiquitination and proteasome-mediated degradation of p53. *Cancer Res* 1996; **56**: 2649–2654.
27. Crook T, Marston NJ, Sara EA, Vousden KH. Transcriptional activation by p53 correlates with suppression of growth but not transformation. *Cell* 1994; **79**: 817–827.
28. Pietenpol JA, Tokino T, Thiagalingam S, El-Deiry WS, Kinzler KW, Vogelstein BS. Sequence-specific transcriptional activation is essential for growth suppression by p53. *Proc Natl Acad Sci U S A* 1994; **91**: 1998–2002.
29. Barak Y, Juven T, Haffner R, Oren M. mdm2 expression is induced by wild-type p53 activity. *EMBO J* 1993; **12**: 461–468.
30. Momand J, Zambetti GP, Olson D, George D, Levine A. The mdm-2 oncogene product forms a complex with the p53 protein and inhibits p53-mediated transactivation. *Cell* 1992; **69**: 1237–1245.
31. Wu X, Bayle JH, Olson D, Levine A. The p53-mdm-2 autoregulatory feedback loop. *Genes Dev* 1993; **7**: 1126–1132.
32. Haupt Y, Maya R, Kazak A, Oren M. Mdm2 promotes the rapid degradation of p53. *Nature* 1997; **387**: 296–299.
33. Kubbutat MHG, Jones SN, Vousden KH. Regulation of p53 stability by Mdm2. *Nature* 1997; **387**: 299–303.
34. Siliciano JD, Canman CE, Taya Y, Sakaguchi E, Appella E, Kastan MB. DNA damage induces phosphorylation of the amino terminus of p53. *Genes Dev* 1997; **11**: 3471–3481.
35. Shieh SY, Ikeda M, Taya Y, Prives C. DNA damage-induced phosphorylation of p53 alleviates inhibition by MDM2. *Cell* 1997; **91**: 325–334.
36. Pomerantz J, Schreiber-Agus N, Liegeois NJ, Silverman A, Alland L, Chin L, Potes J, Chen K, Orlow I, Lee H-W, Cordon-

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Cardo C, DePihno R. The Ink4a tumor suppressor gene product, p19Arf, interacts with MDM2 and neutralizes MDM2's inhibition of p53. *Cell* 1998; **92**: 713–723.
37. Zhang Y, Xiong Y, Yarbrough WG. ARF promotes MDM2 degradation and stabilizes p53: ARF-INK4a locus deletion impairs both the Rb and p53 tumor suppressor pathways. *Cell* 1998; **92**: 725–734.
38. Montes de Oca Luna R, Wagner DS, Lozano G. Rescue of early embryonic lethality in mdm2-deficient mice by deletion of p53. *Nature* 1995; **378**: 203–206.
39. Jones SN, Roe AE, Donehower LA, Bradley A. Rescue of embryonic lethality in Mdm2-deficient mice by absence of p53. *Nature* 1995; **378**: 206–208.
40. Gudas JM, Nguyen H, Klein RC, Katayose D. Differential expression of multiple MDM2 messenger RNAs and proteins in normal and tumorigenic breast epithelial cells. *Clin Cancer Res* 1995; **1**: 71–80.
41. Crescenzi M, Soddu S, Tatò F. Mitotic cycle reactivation in terminally differentiated cells by adenovirus infection. *J Cell Physiol* 1995; **162**: 26–35.

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