 Therapeutic Efficacy of p53 Restoration in Mdm2-Overexpressing Tumors

Qin Li1,2, Yun Zhang1, Adel K. El-Naggar3, Shunbin Xiong1, Peirong Yang1, James G. Jackson1, Gilda Chau1, and Guillermina Lozano1

Abstract

The p53 (TP53) tumor suppressor is the most frequently mutated gene in human cancers. Restoring expression of wild-type p53 has led to tumor growth suppression in a variety of tumor models that are p53 deficient. Other mechanisms, for example, upregulation of Mdm2, exist in tumors to inactivate the p53 pathway. Mdm2, an E3 ubiquitin ligase that targets p53 for proteasomal degradation, is present at high levels in many tumors with wild-type p53. In this study, the effects of restoring p53 activity were probed in Mdm2-overexpressing tumors genetically using animal models. Here, it was demonstrated that elevated levels of Mdm2 and decreased levels of p53 act additively to dampen p53 activity in DNA damage response and tumor development. Our data further indicate that restoration of wild-type p53 expression in Mdm2-overexpressing angiosarcomas results in tumor stasis and regression in some cases. Finally, it was determined that restored p53 suppressed cell proliferation but did not elicit apoptosis in the Mdm2-overexpressing angiosarcomas.

Implications: Restoration of wild-type p53 expression in Mdm2-overexpressing tumors suppresses tumor growth, which represents a potential clinical strategy to treat tumors with high levels of Mdm2.

Visual Overview: http://mcr.aacrjournals.org/content/12/6/901/F1.large.jpg.


Introduction

The tumor suppressor p53 functions as a transcription factor to transactivate a set of genes involved in cell-cycle arrest, cellular senescence, and apoptosis in response to a variety of intra- and extracellular signals, including DNA damage, oncogene activation, and hypoxia. These growth-suppressing activities of p53 thus eliminate cells with transforming potential. Therefore, TP53, is the most mutated gene in human malignancies (1).

Thus, reintroduction of functional p53 into tumors lacking p53 expression was proposed to be an effective therapeutic strategy. Delivery of exogenous wild-type p53 into tumor cells with p53 loss using retroviral vectors led to growth suppression of various tumors in orthotopic mouse models and human patients (2–4). Subsequently, four research groups established genetically engineered mouse models, in which wild-type p53 expression could be restored in tumors lacking functional p53 (5–8). Specifically, our laboratory developed a knock-in mouse carrying a p53<sup>Wt</sup> allele, in which a loxP-flanked PGK-neomycin stop-cassette was inserted into the p53 locus. This hypomorphic allele expresses approximately 8% of p53 levels per allele compared with that of a wild-type p53 allele (8). The expression of p53 can be fully recovered by using Cre recombinase to remove the PGK-neomycin cassette (8). Restoration of p53 expression led to regression of lymphomas, sarcomas, and carcinomas that developed in mice lacking p53 by inducing senescence and/or apoptosis (5–8). However, restoring p53 expression in tumors inheriting a p53<sup>R172H</sup> missense mutation resulted in tumor stasis but not regression due to the dominant-negative effects of p53<sup>R172H</sup> mutant on wild-type p53 (8). Although less effective, restoration of wild-type p53 expression is able to induce antitumor activities even in the presence of mutant p53 (8).

The functions of p53 are also muted in a significantly large number of tumors that lack TP53 alterations (9), suggesting that other mechanisms contribute to silencing tumor-suppressive activity of p53 during tumorigenesis. Extensive studies have indicated that overexpression of murine double minute 2 (Mdm2), a major negative regulator of p53, serves as an alternate means to inactivate the p53 pathway during tumorigenesis (10). Extensive in vitro and in vivo data indicate that Mdm2 negatively regulates p53 function by binding to p53, blocking its transcripational activity, and functioning as an E3 ubiquitin ligase to ubiquitylate p53 and target it for proteasomal degradation (11). Clinical data demonstrate that a large number of human tumors have
elevated Mdm2 levels but lack TP53 mutations, which suggests that Mdm2 overexpression and TP53 mutation are mutually exclusive because either mechanism inactivates the p53 pathway (12). Therefore, drugs to inhibit Mdm2 in an attempt to reactivate antitumor functions of wild-type p53 have been developed. A number of Mdm2 inhibitors, which either disrupt Mdm2–p53 interaction or inhibit Mdm2 E3 ligase activity, induce p53 activities and suppress tumor growth in tumors carrying wild-type TP53 (10).

Accumulating evidence also indicates that a proportion of human tumors exist, which feature both Mdm2 overexpression and loss of wild-type TP53 (13–25). More interestingly, patients with tumors carrying both alterations generally show more malignant disease and poorer prognosis, compared with the ones with tumors bearing either alteration alone (17, 21, 23, 24). These observations suggest that alterations in both Mdm2 and TP53 may confer additional growth advantages to tumor cells. Previously, Jones and colleagues demonstrated that Mdm2 transgenic (Mdm2Tg) mice with an average 4-fold increase in Mdm2 levels compared with wild-type mice developed tumors with 100% penetrance and long latency (26). The tumor onset in Mdm2Tg p53–/– mice occurred much earlier than that in Mdm2Tg mice but showed no difference from that of p53–/– mice (26). Currently, there is no direct evidence showing that elevated Mdm2 levels and dampened TP53 expression additively contribute to tumorigenesis in vivo. Moreover, many human tumors that retain wild-type TP53 have 10- to 50-fold increase in Mdm2 levels (13, 27, 28), which cannot be achieved in the Mdm2Tg mouse model due to embryonic lethality caused by very high levels of Mdm2 (26). Therefore, a mouse model with decreased p53 expression in an Mdm2Tg background better mimics the Mdm2:p53 ratio in human Mdm2-overexpressing tumors with wild-type p53. To this end, we established a mouse model with ectopic expression of Mdm2 and dampened expression of p53 to examine the additive effects of these two alterations on tumorigenesis and subsequently to investigate therapeutic efficacy of p53 restoration in tumors with high Mdm2 levels.

Materials and Methods

Mice

The p53Neo/Neo mice (8) were intercrossed with Mdm2Tg mice (26) and CreER mice (29). All mice were maintained in a genetic background that was >95% C57BL/6J. The mice were bred and maintained in compliance with the Institutional Animal Care and Use Committee of the University of Texas M.D. Anderson Cancer Center.

Magnetic resonance imaging

During MRI, mice were subjected to anesthesia as previously described (8), 5% isoflurane in oxygen to initiate anesthesia and then 2% to 3% isoflurane in oxygen to maintain anesthesia. Respiratory bellows in conjunction with a monitoring and gating system (Small Animal Instruments, Inc.) were used to monitor physiologic conditions of anesthetized mice. The MR images were acquired using a 7 Tesla small animal MR system BioSpec USR70/30 (Bruker Biospin MRI) with a 6 cm imaging gradient. A RF coil with 3.5 cm internal diameter was used for signal excitation and detection. T2-weighted coronal and axial MR image stacks were used to calculate tumor volumes using ParaVision 4.0 software (Bruker Biospin MRI).

Tamoxifen treatment

Tamoxifen (Sigma-Aldrich) was prepared as a 30 mg/mL stock solution with corn oil:ethanol mixture (95:5:4.5, v/v). Tumor-carrying mice received weekly intraperitoneal injection of tamoxifen at a dose of 3 mg/40 g bodyweight. Tamoxifen-treated mice were closely monitored for up to 4 weeks or until moribundness and then sacrificed.

Transplantation

The primary Mdm2Tg p53Neo/Neo CreER angiosarcoma was excised from a euthanized mouse, extensively diced with a scalpel blade, and trypsinized at 37°C for 10 minutes. DMEM with 10% FBS was used to inactivate trypsin and then the mixture was passed through a 40 μm filter to remove cell debris. Tumor cells were resuspended in PBS: Matrigel mixture (2:1, v/v) to a final density 2 × 10⁷/mL at 4°C, following PBS washing. Two million tumor cells in PBS:Matrigel solution (100 μL) were injected subcutaneously into the flanks of C57BL/6J mice. Tumor formation was monitored by palpation. The sizes of these tumors were monitored with digital calipers and tumor volumes were calculated using the equation [(width² × length)/2] as described previously (30).

Histopathology and immunohistochemistry

Tumor tissues collected from mice were processed for histopathologic analysis as previously described (31). Hematoxylin and eosin (H&E) staining was performed on paraffin-embedded sections at the Veterinary Histopathology Laboratory at the University of Texas M.D. Anderson Cancer Center. Immunohistochemical staining was performed as previously described (32), using antibodies for Ki-67 (MM1, Leica Biosystems; 1:200), cleaved caspase-3 (5A1E, Cell Signaling Technology; 1:200), and PML (PGM-3, Santa Cruz Biotechnology; 1:100).

Murine embryonic fibroblast preparation, cell culture, and MTT assay

Murine embryonic fibroblasts (MEF) were established from 13.5 days post coitum embryos as described (33). pBabe-puro–based retroviral vectors were used to transduce Phoenix-eco cells to produce retroviral particles; and then retroviral particles collected from the Phoenix-eco cell medi-um were used to infect MEFS. MEFS infected with these retroviral particles were subjected to screening for puromycin-resistant cells in culture medium with 2 μg/mL puromycin for 4 days. 4-hydroxytamoxifen treatment to MEFS was performed using culture medium containing 1 μmol/L 4-hydroxytamoxifen. To induce p53 activation in MEFS, cells were incubated in culture medium with 1 μg/mL DNA-damaging drug doxorubicin for 4 hours. For MTT assays, cells were incubated in culture medium with 125 μg/mL.
MTT for 3 hours followed by dimethyl sulfoxide incubation for 20 minutes, and the optical absorbance was measured at the wavelength of 550 nm.

**Real-time reverse transcription PCR**

Total RNAs were isolated from MEFs or mouse tissues using TRIzol reagent (Life Technologies) following manufacturer's instructions. Reverse transcription reactions for first-strand cDNA synthesis were performed using the First-Strand cDNA Synthesis Kit with random hexamers as primers (GE Healthcare). Real-time PCR was performed on a 7900HT Fast Real-time PCR system (Applied Biosystems) using iTaq SYBR Green Supermix with ROX or SsoAdvanced SYBR Green Supermix (Bio-Rad). Primer sets used in the real-time PCR probing for Mdm2, Pmaip1 (Noxa), Cdkn1a, Bcl3 (Puma), and Rplp0 were previously described (34). In all quantitative real-time reverse transcription PCR (qRT-PCR) experiments, mRNA expression was normalized to Rplp0 levels.

**Western blotting**

Proteins were extracted from MEFs or homogenized mouse tissues using radioimmunoprecipitation assay (RIPA) buffer with complete proteinase inhibitors (Roche Applied Science). Proteins, resolved in SDS-PAGE and transferred to polyvinyliden difluoride membranes, were incubated with antibodies against p53 (CM5, Vector Laboratories; 1:1,000), Mdm2 (2A10, Calbiochem; 1:1,000), p21 (F-5, Santa Cruz Biotechnology; 1:200), β-actin (AC-74, Sigma-Aldrich; 1:5,000), and γ-tubulin (Sigma-Aldrich; 1:1,000). Membrane-bound proteins were then visualized with ECL Western blotting detection reagent (GE Healthcare), following incubation with appropriate horseradish peroxidase-conjugated secondary antibodies (GE Healthcare).

**Statistical analysis**

All statistical analyses were performed using the GraphPad Prism software (GraphPad Software). Difference between genotypes or treatment groups was analyzed with two-tailed Student t test and difference between survival curves was determined via log-rank (Mantel–Cox) test. A P value of <0.05 was deemed statistically significant.

**Results**

**Mdm2 overexpression and decreased p53 expression additively dampen p53 activities**

The Mdm2Tg mice carrying an integrated Mdm2 cosmid, expressing an average 4-fold of Mdm2 compared with that of wild-type mice, are tumor prone (26), while the p53Neo/Neo mice inheriting a hypomorphic p53 allele have decreased expression of wild-type p53 and are also tumor prone (8). To test whether Mdm2 overexpression and decreased p53 levels will combinatorially affect p53 function, we crossed Mdm2Tg mice (26) with p53Neo/Neo mice to generate Mdm2Tg p53Neo/Neo mice. As shown in Fig. 1A, the Mdm2 transcript levels in the spleens of Mdm2Tg p53Neo/Neo mice were comparable with that of Mdm2Tg mice, but higher than that of wild-type and p53Neo/Neo mice, suggesting that the p53 status does not affect expression of the Mdm2 transgene under physiologic conditions. To compare p53 activity in mice with different genotypes, we tested wild-type, p53Neo/Neo, Mdm2Tg, and Mdm2Tg p53Neo/Neo mice with 6 Gy ionizing radiation. Upon ionizing radiation treatment, less p53 was stabilized in the spleens of Mdm2Tg and p53Neo/Neo mice compared to that of wild-type spleens, and p53 levels in the ionizing radiation-treated Mdm2Tg p53Neo/Neo mouse spleens were even lower than that of Mdm2Tg and p53Neo/Neo spleens (Fig. 1B). Induction of specific p53 target genes p21 and Puma by ionizing radiation was significantly reduced in Mdm2Tg and p53Neo/Neo spleens and further dampened in Mdm2Tg p53Neo/Neo mouse spleens compared to wild-type spleens (Fig. 1C). These data together indicate that Mdm2 overexpression and decreased p53 levels additively compromise p53 function in the DNA damage response.

In addition, Mdm2Tg p53Neo/Neo mice showed a significant shorter tumor latency compared with Mdm2Tg and p53Neo/Neo mice (Fig. 1D), indicating that Mdm2 overexpression and p53 loss cooperate to promote a more aggressive phenotype. The most common tumor types observed in these mice were lymphomas and sarcomas (Table 1).

**p53 restoration suppresses the growth of Mdm2Tg p53Neo/Neo CreER MEFs**

To examine effects of p53 restoration in Mdm2-overexpressing cells, we restored p53 expression in Mdm2Tg p53Neo/Neo CreER MEFs using 4-hydroxysteromuxifen to induce the DNA recombinase activity of CreER. Upon 4-hydroxysteromuxifen treatment, the mRNA levels of p53 increased by 8-fold in the Mdm2Tg p53Neo/Neo CreER MEFs compared with that of the vehicle-treated control cells (Fig. 2A). In MTT assays, 4-hydroxysteromuxifen treatment significantly suppressed the growth of Mdm2Tg p53Neo/Neo CreER MEFs compared with the vehicle-treated control (Fig. 2B), suggesting a correlation between restoration of p53 expression and suppression of cell growth. We then investigated whether the restoration of p53 expression would lead to enhanced p53 function in response to DNA damage. After treatment with the DNA-damaging agent doxorubicin, expression of p53 target genes p21 and Noxa in the 4-hydroxysteromuxifen-treated Mdm2Tg p53Neo/Neo CreER MEFs was significantly higher than that of controls (Fig. 2C). These data collectively indicate that restoration of p53 expression in Mdm2-overexpressing cells can lead to growth suppression and enhanced p53 function, which is amplified by DNA damage.

To further investigate the effects of restoring p53 under oncogene activation conditions, we infected Mdm2Tg p53Neo/Neo CreER MEFs with an H-RasG12V retrovirus. Ectopic expression of H-RasG12V led to stabilization of p53 in these MEFs, and further restoration of p53 expression by 4-hydroxysteromuxifen treatment resulted in higher p53 protein levels in H-RasG12V-infected Mdm2Tg p53Neo/Neo CreER MEFs compared with that of the control cells (Fig. 2D). Interestingly, H-RasG12V levels decreased upon p53
restoration by 4-hydroxytamoxifen treatment for unknown reasons. One plausible explanation for this unexpected observation is that accumulation of restored p53 strongly selected against cells with high levels of H-RasG12V protein. However, we cannot rule out the possibility that high levels of p53 downregulate H-RasG12V expression via yet unknown mechanisms, as it has been reported that exogenous wild-type p53 suppresses the mRNA expression of a mutant H-Ras gene in bladder cancer cells (35). Further investigation is warranted to better understand this interesting observation.

Figure 1. Additive effects of Mdm2 overexpression and decreased p53 levels on suppression of p53 activities. A, qRT-PCR analysis of Mdm2 expression in the spleens of 4- to 6-week-old mice with wild-type (WT; n = 5), p53Neo/Neo (N/N; n = 4), Mdm2Tg (M2Tg; n = 3), and Mdm2Tg p53Neo/Neo (M2Tg N/N; n = 4) genotypes. B, Western blot analysis of p53 levels in the spleens of ionizing radiation (IR; 6 Gy)-treated 4- to 6-week-old mice with wild-type (WT), p53Neo/Neo (N/N), Mdm2Tg (M2Tg), and Mdm2Tg p53Neo/Neo (M2Tg N/N) genotypes. For each genotype, protein lysate samples from 3 mouse spleens were pooled together. Protein lysate from an Mdm2+/C0 p53+/C0 spleen served as negative control. Percentages at the bottom indicate the relative p53 levels in each genotype in comparison with that of wild-type. C, qRT-PCR analysis of expression of p53 target genes p21 and Puma in the spleens of 4- to 6-week-old wild-type (WT; n = 5), p53Neo/Neo (N/N; n = 4), Mdm2Tg (M2Tg; n = 3), and Mdm2Tg p53Neo/Neo (M2Tg N/N; n = 4) mice with or without ionizing radiation treatment (6 Gy). Data were normalized to expression in a wild-type spleen and represent a mean ± SEM. D, tumor-free survival curves of Mdm2Tg (n = 39), p53Neo/Neo (n = 21), and Mdm2Tg p53Neo/Neo (n = 53) mice.

Table 1. Tumor spectra of p53Neo/Neo, Mdm2Tg and Mdm2Tg p53Neo/Neo mice

<table>
<thead>
<tr>
<th>Tumor types</th>
<th>p53Neo/Neo (n = 19)</th>
<th>Mdm2Tg (n = 24)</th>
<th>Mdm2Tg p53Neo/Neo (n = 44)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lymphoma</td>
<td>7 (28.0%)</td>
<td>16 (61.5%)</td>
<td>20 (35.7%)</td>
</tr>
<tr>
<td>Sarcoma</td>
<td>15 (60.0%)</td>
<td>4 (15.4%)</td>
<td>28 (50%)</td>
</tr>
<tr>
<td>Angiosarcoma</td>
<td>8</td>
<td>3</td>
<td>15</td>
</tr>
<tr>
<td>Spindle-cell sarcoma</td>
<td>5</td>
<td></td>
<td>11</td>
</tr>
<tr>
<td>Other sarcomas</td>
<td>2</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Other</td>
<td>3 (12%)</td>
<td>6 (23.1%)</td>
<td>8 (14.3%)</td>
</tr>
<tr>
<td>Total</td>
<td>25</td>
<td>26</td>
<td>56</td>
</tr>
</tbody>
</table>
The expression of p53 target genes p21 and Noxa was induced upon restoration of p53 by 4-hydroxytamoxifen in the Mdm2Tg p53Neo/Neo CreER MEFs regardless of H-RasG12V infection (Fig. 2E). Interestingly, upon 4-hydroxytamoxifen treatment, the expression levels of p21 and Noxa were significantly higher in the H-RasG12V-infected MEFs than that of control cells (Fig. 2E), suggesting that oncogene activation potentiates function of restored p53 in the Mdm2-overexpressing cells. As illustrated with MTT assays, H-RasG12V-infected Mdm2Tg p53Neo/Neo CreER MEFs upon 4-hydroxytamoxifen treatment grew significantly slower than the vehicle-treated cells (Fig. 2F). In addition,
in the presence of 4-hydroxytamoxifen, the growth of H-RasG12V-infected MEFs was also significantly slower than that of the cells without H-RasG12V infection (Fig. 2F). These data together indicate that reintroducing p53 expression in oncogene-overexpressing cells may confer more potent growth-suppressing activities in an Mdm2-overexpressing background.

**p53 restoration leads to growth suppression in spontaneous Mdm2-overexpressing angiosarcomas**

To examine the therapeutic effects of p53 restoration on tumors in vivo, we established a mouse cohort with both Mdm2Tg p53Neo/Neo CreER and the Mdm2Tg p53Neo/Neo genotypes. The tumor-free survival curves of untreated mice from both genotypes overlap with each other (Supplementary Fig. S1), indicating that the presence of CreER transgene did not affect tumor development in the Mdm2Tg p53Neo/Neo CreER group. Tumor formation in mice of both genotypes was closely monitored with MRI biweekly. After tumors reached a certain size (>50 mm³), a proportion of tumor-bearing mice underwent weekly tamoxifen treatment for up to 4 weeks. Three major tumor types were captured in the tamoxifen-treated group, and included angiosarcomas, spindle-cell sarcomas, and lymphomas. To examine the effects of p53 restoration in these tumors, we first analyzed the recombination rate of p53Neo allele in the Mdm2Tg p53Neo/Neo CreER tumors upon tamoxifen treatment. An average of 7% recombination rate was observed in the lymphomas after tamoxifen treatment (Supplementary Fig. S2). Three treated Mdm2Tg p53Neo/Neo CreER spindle-cell sarcomas showed highly variable recombination rates ranging from 97% and 61% (Supplementary Fig. S2). The Mdm2Tg p53Neo/Neo CreER angiosarcomas had a uniform recombination rate with an average of 33% upon 4 doses of tamoxifen treatment (Supplementary Fig. S2). Therefore, we focused on these angiosarcomas and used them as a model system to study the effects of p53 restoration on Mdm2-overexpressing tumors (Supplementary Table S1).

Upon tamoxifen treatment, the Mdm2Tg p53Neo/Neo CreER mice carrying angiosarcomas survived significantly longer than the angiosarcoma-bearing Mdm2Tg p53Neo/Neo mice (Fig. 3A), suggesting that p53 restoration can prolong the survival of these tumor-carrying mice. We then compared the changes in tumor volumes (posttreatment/pretreatment ratio) between the Mdm2Tg p53Neo/Neo CreER and Mdm2Tg p53Neo/Neo groups. Mdm2Tg p53Neo/Neo CreER angiosarcomas grew slower than the Mdm2Tg p53Neo/Neo angiosarcomas upon tamoxifen treatment (Fig. 3B and C). To test the possibility that differences in posttreatment/pretreatment ratios between these two mouse genotypes were due to the differences in initial tumor volumes, we compared the pretreatment volumes of angiosarcomas between both genotypes. There were no significant differences in tumor volumes between Mdm2Tg p53Neo/Neo CreER and Mdm2Tg p53Neo/Neo angiosarcomas at the time that tamoxifen treatment started (Supplementary Fig. S3), suggesting that p53 restoration by tamoxifen treatment led to growth suppression in the Mdm2-overexpressing angiosarcomas.

**p53 restoration slows the growth of Mdm2-overexpressing angiosarcomas in a syngeneic mouse model**

We then aimed to determine what type of response occurred upon restoring p53 in the Mdm2-overexpressing angiosarcomas. Although higher average levels of p53 target gene, for example, p21 was observed in Mdm2Tg p53Neo/Neo CreER angiosarcomas compared with that of the Mdm2Tg p53Neo/Neo angiosarcomas in response to tamoxifen administration, the difference between the two groups were not statistically significant (Supplementary Fig. S4). One plausible explanation for this unexpected observation is the heterogeneity of tumors, which might contribute to intergroup variation and lack of differences between groups. Therefore, we established a syngeneic tumor mouse model of angiosarcomas. Briefly, a spontaneous Mdm2Tg p53Neo/Neo CreER angiosarcoma was processed into single-cell suspension, and injected into the subcutaneous flank regions of multiple syngeneic mice. In this transplant model, more than 90% of p53Neo alleles in the transplant tumors were recombined upon tamoxifen administration (Supplementary Fig. S5). In agreement with aforementioned observations, the tamoxifen-treated angiosarcomas grew significantly slower than the vehicle-treated controls (Fig. 4A). The tumor doubling time of Mdm2Tg p53Neo/Neo CreER angiosarcomas upon tamoxifen administration (10.9 days) was much longer than that of Mdm2Tg p53Neo/Neo CreER angiosarcomas undergoing vehicle treatment (4.5 days; Fig. 4B). This tumor growth suppression was apparently not caused by independent effects of tamoxifen, as tamoxifen injection did not alter tumor growth of the Mdm2Tg p53Neo/Neo angiosarcomas (Supplementary Fig. S6). These data collectively indicate a positive correlation between p53 restoration and tumor growth suppression in the transplant tumor model.

Restoration of p53 expression by tamoxifen in these transplanted angiosarcomas led to accumulation of p53 and p21 proteins compared with control tumors (Fig. 4C). We then examined the induction of p53 target gene expression in the transplanted Mdm2Tg p53Neo/Neo CreER angiosarcomas upon tamoxifen injection. In comparison with the vehicle-treated tumors, tumors treated with tamoxifen expressed significantly higher levels of p53 targets p21 and Puma (Fig. 4D). These data together indicate that tamoxifen administration in the transplanted Mdm2Tg p53Neo/Neo CreER angiosarcomas restored expression of functional p53 that led to elicitation of gene expression downstream to p53.

**Suppression of cell proliferation but not induction of apoptosis is triggered by p53 restoration in Mdm2-overexpressing angiosarcomas**

To examine the biologic and cellular effects of p53 restoration in the Mdm2Tg p53Neo/Neo CreER angiosarcomas, we analyzed the proliferation index by immunohistochemistry in tumors with chronic tamoxifen treatment. Upon tamoxifen treatment, an average of 48% of cells were positive...
for proliferation marker Ki-67 in Mdm2Tg p53NeoNeo CreER and Mdm2Tg p53NeoNeo mice with tamoxifen treatment. A, survival curves of Mdm2Tg p53NeoNeo CreER and Mdm2Tg p53NeoNeo mice with angiosarcomas upon tamoxifen treatment. B, tumor volume changes (posttreatment volume/pretreatment volume ratios) of angiosarcomas in Mdm2Tg p53NeoNeo CreER and Mdm2Tg p53NeoNeo mice during tamoxifen treatment. C, MRI images and changes in tumor volumes of two representative angiosarcomas (1 Mdm2Tg p53NeoNeo CreER and 1 Mdm2Tg p53NeoNeo during tamoxifen treatment. Day 0, the day when tamoxifen treatment started; Day 26, 28 days after initial tamoxifen treatment, etc.

Figure 3. Survival and tumor volume changes in angiosarcomas of Mdm2Tg p53NeoNeo CreER and Mdm2Tg p53NeoNeo mice with tamoxifen treatment. A, survival curves of Mdm2Tg p53NeoNeo CreER and Mdm2Tg p53NeoNeo mice with angiosarcomas upon tamoxifen treatment. B, tumor volume changes (posttreatment volume/pretreatment volume ratios) of angiosarcomas in Mdm2Tg p53NeoNeo CreER and Mdm2Tg p53NeoNeo mice during tamoxifen treatment. C, MRI images and changes in tumor volumes of two representative angiosarcomas (1 Mdm2Tg p53NeoNeo CreER and 1 Mdm2Tg p53NeoNeo during tamoxifen treatment. Day 0, the day when tamoxifen treatment started; Day 26, 28 days after initial tamoxifen treatment, etc.

We also analyzed the expression of cellular senescence marker PML in the tamoxifen-treated spontaneous angiosarcomas. In Mdm2Tg p53NeoNeo CreER angiosarcomas, 3 of 9 tumors treated with multiple doses of tamoxifen and 4 of 5 treated with one dose of tamoxifen showed positive for immunohistochemical staining of senescence marker PML; and no positive PML staining was observed in any tamoxifen-treated Mdm2Tg p53NeoNeo angiosarcomas (Fig. 5D–F). Therefore, our data suggest that restoration of p53 expression induces a senescence response at least in a proportion of the Mdm2-overexpressing angiosarcomas.

To analyze the apoptotic effects of p53 restoration in the Mdm2Tg p53NeoNeo CreER tumors, we compared the cleaved caspase-3 levels by immunohistochemistry between the tamoxifen-treated Mdm2Tg p53NeoNeo CreER and Mdm2Tg p53NeoNeo CreER angiosarcomas.
p53Neo/Neo angiosarcomas. Interestingly, no apoptosis was induced in the Mdm2Tg p53Neo/Neo CreER angiosarcomas with either chronic or acute tamoxifen treatment compared with Mdm2Tg p53Neo/Neo angiosarcomas (Fig. 5G–I), indicating that reintroduction of p53 in these tumors does not induce an apoptotic response mediated by cleaved caspase-3.

Discussion

In this study, we established a mouse model with high levels of Mdm2 (via transgene expression) and decreased levels of p53 (via a hypomorphic p53 allele). These mice showed dampened p53 levels and activities in response to DNA-damaging agents, compared with mice with either high Mdm2 levels or decreased p53 levels. In addition, Mdm2Tg p53Neo/Neo mice developed tumors significantly earlier than Mdm2Tg and p53Neo/Neo mice. These data were consistent with the previous reports that loss of p53 in an Mdm2Tg background evidently shortened tumor latency in mice (26). However, in the previous study, the tumor-free survival of Mdm2Tg p53−/− mice overlapped with that of p53−/− mice (26), leaving the question whether high Mdm2 and decreased p53 levels additively contribute to tumor formation unanswered. Our data here clearly showed that a dose-dependent tumor-suppressing function of p53. Strikingly, Mdm2Tg p53Neo/Neo mice (median tumor-free survival equals 348 days) did not develop tumors as early as p53−/− mice (median tumor-free survival is approximately 160 days) described previously (35), suggesting that even extremely low levels of p53 are sufficient to execute antitumor function and to delay tumor formation.

Restoration of p53 in Mdm2Tg p53Neo/Neo CreER MEFs resulted in increased p53 activity and suppression of cell proliferation in comparison with vehicle-treated control cells. p53 restoration led to elevated induction of p53 target genes in response to doxorubicin treatment. These observations indicate that the restored p53 possesses the ability to function in growth control and DNA damage response even in an Mdm2-overexpressing scenario. We also overexpressed an oncogene H-RasG12V in Mdm2Tg p53Neo/Neo CreER MEFs to mimic oncogene activation of p53. Consistent with previous studies (36), ectopic expression of H-RasG12V led to accumulation of p53 proteins compared with cells without H-RasG12V. Of note, p53 restoration enhanced p53 accumulation in the H-RasG12V-overexpressing Mdm2Tg
p53Neo/Neo CreER MEFs. Intriguingly, p53-restoration–induced expression of p53 target genes was significantly stronger in the H-RasG12V-infected cells compared with that of control cells. In addition, we noticed that restoration of p53 resulted in slower growth in the H-RasG12V–infected Mdm2Tg p53Neo/Neo CreER angiosarcomas compared with that of control MEFs. These data suggest that oncogene activation potentiates p53 activity even in presence of high levels of Mdm2.

Tamoxifen-treatment prolonged the survival of the Mdm2Tg p53Neo/Neo CreER mice bearing angiosarcomas in comparison with that of the Mdm2Tg p53Neo/Neo CreER MEFs. These data suggest that oncoprotein activation potentiates p53 activity even in presence of high levels of Mdm2.

Figure 5. Reduced cell proliferation and induced cellular senescence in Mdm2Tg p53Neo/Neo CreER angiosarcomas upon tamoxifen treatment. These data indicate that p53 restoration is able to suppress the growth of Mdm2-overexpressing angiosarcomas.

p53 exerts its tumor suppressive activities by inducing apoptosis, cell-cycle arrest, and senescence programs (37). Although the mechanisms are not entirely clear, different tissues respond differently to a variety of stimuli (37). In the current study, p53 restoration led to reduced Ki-67 staining but no increase in cleaved caspase-3 staining in tamoxifen-treated Mdm2Tg p53Neo/Neo CreER angiosarcomas, suggesting that restored p53 inhibited cell proliferation. This observation is consistent with previous studies showing that restoration of p53 in sarcomas, including angiosarcomas,
results in cell-cycle arrest but not apoptosis (8). These data indicate that the intrinsic characteristics of angiosarcomas determine the cell-cycle arrest as the default program induced by p53 restoration. Proliferation arrest is now an established mechanism by which reintroduction of p53 affects numerous cancers (6–8), and converting this arrest phenotype to an apoptotic response will be of critical importance for clinical therapies.

Pharmacologically restoring p53 function in Mdm2-overexpressing tumors includes identifying Mdm2 inhibitors. However, the nature of Mdm2 inhibitors determines the limitations of these pharmaceuticals in tumor treatments. Because the therapeutic efficacy of Mdm2 inhibitors is dependent on the presence of wild-type p53 in tumors (10), treatments with the Mdm2 inhibitors are unlikely to induce antitumor effects in tumors with concurrent Mdm2 overexpression and p53 loss. Hence, alternative approaches, for example, p53 gene delivery, are in great demand for treatment of Mdm2-overexpressing tumors, especially the ones with loss of wild-type p53. Previous studies have demonstrated that ectopically expressing wild-type p53 via gene transfer confers antitumor effects in both preclinical and clinical studies (2–4). However, it is natural to speculate that this approach may not be effective for treatment of Mdm2-overexpressing tumors, as high levels of Mdm2 can readily degrade p53 and suppress p53 transcriptional activity. Strikingly, our present study indicates that restoring expression of endogenous p53 in tumors with high levels of Mdm2 was able to suppress tumor growth in an animal model. Therefore, our study serves as a proof-of-principle for p53 gene therapy of Mdm2-overexpressing tumors.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors' Contributions
Conception and design: Q. Li, G. Lozano
Development of methodology: Q. Li, J.G. Jackson
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): Q. Li, Y. Zhang, J.G. Jackson
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): Q. Li, S. Xiong
Writing, review, and/or revision of the manuscript: Q. Li, Y. Zhang, A.K. El-Naggar, G. Lozano
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): Q. Li, S. Xiong, P. Yang, J.G. Jackson, G. Chau
Study supervision: G. Lozano

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