p53-dependent Down-regulation of Telomerase Is Mediated by p21\textsuperscript{waf1}\textsuperscript{*}

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Inactivation of p53 and activation of telomerase occur in the majority of human cancers, raising the possibility of a link between these two pathways. Overexpression of wild-type p53 down-regulates the enzymatic activity of telomerase in various cancer cell lines through transcriptional repression of its catalytic subunit, human telomerase reverse transcriptase (hTERT). In this study, we re-evaluated the role of p53 in telomerase regulation using isogenic cell lines expressing physiological levels of p53. We demonstrate that endogenous wild-type p53 was able to down-regulate telomerase activity, hTERT mRNA levels, and promoter activity; however, the ability to repress hTERT expression was found to be cell type-specific. The integrity of the DNA-binding core domain, the N-terminal transactivation domain, and the C-terminal oligomerization domains of p53 was essential for hTERT promoter repression, whereas the proline-rich domain and the extreme C terminus were not required. Southwestern and chromatin immunoprecipitation experiments demonstrated lack of p53 binding to the hTERT promoter, raising the possibility of an indirect repressive mechanism. The down-regulation of hTERT promoter activity was abolished by a dominant-negative E2F1 mutant. Mutational analysis identified a specific E2F site responsible for p53-mediated repression. Knockdown of the key p53 transcriptional target, p21, was sufficient to eliminate the p53-dependent repression of hTERT. Inactivation of the Rb family using either viral oncoproteins or RNA interference attenuated the repression. Inhibition of histone deacetylases also interfered with the repression of hTERT by p53. Therefore, our results suggest that repression of hTERT by endogenous p53 is mediated by p21 and E2F.

Telomerase, a specialized RNA-directed DNA polymerase that extends telomeres at the end of eukaryotic chromosomes, has been implicated in aging, immortalization, and transformation. The human telomerase complex is composed of a catalytic subunit (hTERT)\textsuperscript{1} with a reverse transcriptase activity (1) and an RNA-containing subunit (human telomerase RNA) (2) that is used as a template for extending telomere length. Telomerase activity is repressed in most normal human somatic tissues, whereas the enzyme is active in ~90% of human cancers (3). However, the mechanism through which telomerase is reactivated in the process of carcinogenesis remains unclear. Telomerase enzymatic activity can be regulated at multiple levels, including hTERT transcription, alternative splicing, chaperone-mediated folding, phosphorylation, and nuclear translation; however, the major control mechanism of telomerase regulation seems to be at the level of hTERT transcription (for a review of telomerase regulation, see Ref. 4 and references therein).

The tumor suppressor gene p53 is a sequence-specific transcription factor that can mediate many downstream effects such as growth arrest and apoptosis through activation or repression of its target genes (5). p53 is the most frequently altered gene in human cancers. Mutations and deletions of p53 are found in over half of human primary tumors (6). Absence of functional p53 allows cellular immortalization and predisposes cells to neoplastic transformation (7).

Several studies have been conducted in an attempt to correlate the status of p53 and telomerase activity during carcinogenesis. p53-null mouse embryonic fibroblasts exhibit increased (>3-fold) basal levels of telomerase activity relative to those present in matched early passage fibroblasts derived from p53 wild-type-expressing embryos (8). Moreover, elevation of p53 protein levels, which is usually associated with inactivating p53 mutations, is correlated with telomerase expression in cervical cancer (9), breast cancer (10), non-small cell lung cancer (11), and ovarian cancers (12).

Overexpression of wild-type p53 was shown to down-regulate telomerase enzymatic activity in a number of cancer cell lines independent of its effects on growth arrest and apoptosis (13). This observation was attributed to transcriptional repression of hTERT by wild-type p53 since it was preceded by down-regulation of hTERT mRNA (13). This conclusion was supported by promoter activity studies demonstrating the ability of p53 to repress hTERT promoter-reporter constructs. Mapping studies showed that mutation of all five Sp1 transcription factor-binding sites within the core hTERT promoter results in strong down-regulation of the reporter activity, which cannot be further repressed by p53 (14). In an additional study, activation of exogenous temperature-sensitive p53 in BL41 Burkitt’s lymphoma cells triggered rapid down-regulation of hTERT mRNA expression independent of the induction of the p53 target gene transcriptase; siRNA, small interfering RNA; RNAi, RNA interference; RT, reverse transcription; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; UTR, untranslated region; PBS, phosphate-buffered saline.
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Mitomycin C treatment of three breast cancer cell lines results in strong down-regulation of hTERT only in MCF7 cells, which express endogenous wild-type p53, but not in T-47D cells carrying mutant p53 and in p53-null MDA-MB-157 cells. Wild-type p53 inhibits Sp1 binding to the hTERT proximal promoter in a gel shift assay with purified proteins. The ability of p53 and Sp1 to form a complex was shown by co-immunoprecipitation (15). These studies suggest a mechanism of hTERT repression in which p53 binds to Sp1 and renders it inaccessible to hTERT promoter activation (15).

However, the physiological relevance of these findings has been questioned because most of these studies were based on non-physiological overexpression of p53 or lacked isogenic controls (16). In this study, we therefore re-examined the role of p53 in telomerase repression using proper isogenic controls and in lines expressing physiological levels of p53. Our results demonstrate that endogenous p53 represses telomerase in a cell type-specific manner. This p53-induced repression occurs through an indirect mechanism and is mediated by the p21/E2F pathway.

EXPERIMENTAL PROCEDURES

Cell Lines—Human non-small cell lung cancer cell line H1299 and prostate cancer cell line LNCaP were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum and antibiotics. The human breast cancer cell line MCF7 stably expresses small interfering RNA (siRNA) targeting p53 and its vector control line were a gift from Dr. R. Agami (Netherlands Cancer Institute). The RKO colon carcinoma cell line and its derivative overexpressing E6 were a gift from Dr. C. Huris (National Institutes of Health). HT1080 fibrosarcoma cells were a gift from Dr. M. Brandeis (Hebrew University, Jerusalem, Israel). Ecotropic Phoenix retrovirus-producing cells were from American Type Culture Collection. MCF7, RKO, Phoenix cells and p53-null MDA-MB-157 cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, 2 mM l-glutamine, and antibiotics. The HCT-116 colon carcinoma cell line and its p53-null derivative was a gift from Dr. D. Vogelstein (The Johns Hopkins University, Baltimore, MD) (17). They were maintained in McCoy’s medium supplemented with 10% fetal calf serum and antibiotics. All cell lines were grown at 37 °C in a humidified atmosphere of 5% CO2 in air.

Plasmids—The reporter construct containing the p21 promoter (pGL3-Waf1), the expression vectors for a dominant-negative p53 peptide (p53-DD) (18), a p53 deletion mutant lacking the proline-rich domain (62–91del), and wild-type E7 and its deletion mutant lacking the pocket protein-binding region (E7del21–35) were a gift from Dr. M. Oren (Weizmann Institute). The pBabe-GSE56-puro retroviral construct, and pBabe-GSE55-puro lentivirus construct were a gift from Dr. A. V. Gertler (University of Cleveland, OH) (19). pBabe-E1A was a gift from Dr. D. Peepers (Netherlands Cancer Institute). The generation of the reporter constructs containing different lengths of the hTERT promoter and construction of point mutations in the E- and MT-boxes were as described (20, 21).

The additional point mutations of the hTERT promoter were generated on the template of pGL3-core-pHTERT with a QuikChange XL kit (Stratagene) using the following primers (only the sense primer is shown; mutations are in lowercase; and the numbers indicate positions); mutation of the E2F site (E2F-I), 5′-ACCGCTCAGCCGCTCCCCAGCCAAGCACGACGGCCGAGG-3′; mutation of the Sp1 site, 5′-CCGGGAAATCCACACTCCACACCTCC-3′; and for mutation of the E2F site (E2F-II), 5′-CTCCCTTGCCGCGGATGTTCGGCAG-3′. The M6 reporter (point mutation of the most proximal Sp1 site) was constructed by subcloning the SacII and XhoI inserts from the mt5Sp1 plasmid into wild-type pGL3-core-pHTERT digested with the same enzymes.

Expression plasmids for wild-type human p53 and mutants L22Q/H11001, L22Q/H11002, L137V/H11003, and L22Q/H11002/H11001 were prepared as described (25). The following 19-bp sequences were targeted: for p21 (p21), GGACATTGAGAGCTCTGCAC; for mouse p53 (mp53), GCCAGAGGGTCTGCCCGTCC; and for the p53 control mutated sequence (p53-mut), GACTCCCGTTGTAATCTAC.

Western Blots—For Western blotting of p53 and p21 proteins, 50 µg of total cell lysate was separated on 12.5% SDS-PAGE and transferred to a nitrocellulose membrane. Membranes were blocked overnight in 5% dry skimmed milk in PBS (pH 7.2) for 12 h, followed by PBS, 0.1% Tween (pH 7.2), after which the membranes were changed. Fresh viral suspensions were added after a 24-h interval for an additional 12 h. For production of stable RNA interference (RNAi) against p21 or mouse p63 in MCF7 cells, pSuper-Retro-Hygro was used. Following infection, cells were selected with 1 mg/ml hygromycin for 1 week. For production of HT1080 and LNCaP cells with inactivated p53, the pBabe-GSE56-puro retroviral construct was used. Following infection, cells were selected with 1 µg/ml puromycin for 1 week.

Southern Blot Analysis—DNA samples (300 ng of each) were applied to a nitrocellulose membrane and cross-linked to the membrane by UV light at 120 J. The membrane was washed twice with PBS and blocked with 5% milk in PBS for 3 h. Nuclear extracts were prepared as described (26) and diluted to a concentration of 0.5 mg/ml protein in binding buffer (12.5 mM Tris-HCl, pH 7.9, 75 mM KCl, 0.5 mM dithiothreitol, 10% glycerol, 0.25 mM EDTA, 0.2 mM phenylmethylsulfonyl fluoride, 10 µg/ml leupeptin, 1 µg/ml pepstatin, 10 µg/ml aprotinin, 10 µg/ml salmon sperm DNA, and 1 µg/ml pGL3 plasmid DNA for blocking). The diluted nuclear extract was incubated with the membrane overnight at 4 °C. Following washing, the blots were probed with anti-p53 polyclonal antibody, washed with PBS/Tween, and probed with anti-rabbit secondary antibody, and developed using the enhanced chemiluminescence ECL kit.

Chromatin Immunoprecipitation Analysis—Chromatin immunoprecipitation was performed as described previously (27). The primers used for the detection of hTERT promoter sequences that amplify region −217 to +119 relative to the transcription initiation site were 5′-CAGCGCGGCTCCCTGGATGGA-3′ and 3′-CAGCGCGGAGCGCGCGG-3′. The primers used for the detection of p53 sequences that amplify the region near the p53-biding site were 5′-GACCTCTT- GTCCCCCAGAGGATCTCTTG-3′ and 5′-TTATGACGACGAC-3′.

Transfections and Reporter Assays—Cells were grown in complete medium and replated at 3 × 104 cells/well in a 24-well plate 16–24 h before transfection. For reporter gene assay, cells were transfected by using FuGENE 6 transfection reagent (Roche Applied Science) and 250 ng of Rb RNAi, 250 ng of p107 RNAi, and 700 ng of p120 RNAi were cotransfected with the reporter. The targeted sequences are as follows: for p130, CACUUGGGCCUGCAGGAGAUUCCGTT, for Rb, CACUACUACAGUAGGCUAGTT, and for p73, CACAGUGAUGAAGAAGUCC.

Reverse Transcription (RT)-PCR and Telomerase Activity Assays—Total RNA was isolated using the RNeasy kit (Qiagen Inc.), and 2 µg was reverse-transcribed using Moloney murine leukemia virus reverse transcriptase (Promega) and random hexamer primers (Roche Applied Science). Real-time quantitative RT-PCR for total hTERT mRNA was performed using the Assays-on-Demand TaqMan kit (Applied Biosystems) on an ABI 7000 instrument (Applied Biosystems). The values for hTERT were normalized to the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) housekeeping control. The primer sequences for specific qRT-PCR are: p21 primers were PCMV- CTCGACCATTTGATCTATTTGCT; and p21 primers were 5′-TGTCGCTCAGTGACCTCTATTGCAG-3′. For specific detection of full-length hTERT by semiquantitative PCR, the following primers were used: 5′-TGTCGCTCAGTGACCTCTATTGCAG-3′ and 5′-TCGGTGCTGACCTCTATTGCAG-3′. The forward primer is directed to the a-splice site so that the a-spliced form is not amplified. Similarly, the design of the reverse primer prevents amplification of the β-spliced form. PCR was performed in the presence of [32P]dCTP and reaction...
products were detected by autoradiography. Telomerase enzymatic activity was measured using the real-time telomeric repeat amplification protocol essentially as described (28).

RESULTS

p53 Represses Telomerase Activity and hTERT mRNA—To examine the role of endogenous wild-type p53 in telomerase regulation, we used several isogenic cell line pairs specifically differing in their p53 status. To this end, MCF7 breast cancer cells containing wild-type p53 and their derivatives stably expressing p53 RNAi were used (25). As demonstrated in Fig. 1A, induction of the p53 protein following doxorubicin treatment was strongly suppressed in RNAi-containing cells (MCF7-p53i) compared with cells expressing the vector control (MCF7-vector). As shown in Fig. 1B, following 48 h of doxorubicin treatment, a 10-fold down-regulation of telomerase activity was observed in control cells. This inhibition was largely p53-dependent, as only a 2-fold reduction was detected in MCF7-p53i cells.

Next, we examined the levels of hTERT mRNA using real-time quantitative RT-PCR. A significant p53-dependent reduction of hTERT mRNA could be detected as early as 18 h post-treatment (Fig. 1C). The long lag between mRNA down-regulation and the decrease in telomerase enzymatic activity is consistent with the reported long half-life of the latter (29). hTERT was shown to have a complex splicing pattern, which gives rise to at least four isoforms (full-length, a, b, and p7), with only the full-length form catalytically active (30). Since all alternatively spliced forms of hTERT were detected in our experiments, we used hTERT primers specific for this form instead of the other forms (Fig. 1D).

To corroborate the ability of endogenous p53 to down-regulate hTERT expression in an additional isogenic system, we used LNCaP prostate cancer cells containing wild-type p53 and their derivatives in which p53 was inactivated using the dominant-negative GSE56 peptide (31). Following doxorubicin treatment, we observed strong p53-dependent repression of hTERT mRNA (Fig. 1E). Of note, the phenomenon of p53-dependent repression of hTERT mRNA was not general to all the cell lines examined. In a number of isogenic cell lines, the reduction of hTERT mRNA following drug treatment was independent of the p53 status (Table I).

p53 Represses the Transcriptional Activity of the hTERT Promoter—To examine whether p53-mediated down-regulation of hTERT mRNA is due to promoter repression, we performed a series of hTERT promoter-luciferase gene reporter assays (Fig. 2A). As demonstrated in Fig. 2B, wild-type p53 repressed the hTERT promoter constructs of different lengths in a dose-dependent manner when cotransfected into p53-null H1299 cells. The core promoter was sufficient to respond to the p53-mediated repression. To avoid the effects of exogenous p53 overexpression and to extend the analysis to another cell type, we assessed the effect of endogenous p53 by inactivating it using the dominant-negative peptide p53-DD (18). The transfection process itself stabilized and activated endogenous p53 (data not shown); and thus, the reporter activity in the absence of p53-DD reflects the effect of activated p53. Inactivation of p53 by cotransfection with p53-DD strongly reduced the promoter activity of a known p53 transactivation target, p21 

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Mapping of p53 Domains Required for Telomerase Repression—To map the domains of p53 necessary for telomerase repression, H1299 cells were cotransfected with the core hTERT promoter-luciferase reporter construct together with various p53 mutants. Similar expression levels of the different p53 forms were confirmed by Western blotting (data not shown). Fig. 3 demonstrates that the most frequent naturally
TABLE I
Repression of hTERT by endogenous wild-type p53 is cell type-specific

Parental cells containing endogenous wild type-p53 and their derivatives with inactivated p53 were treated with 0.4 μM doxorubicin for 24 or 48 h, and hTERT mRNA levels were determined by real-time quantitative RT-PCR. Following normalization to the GAPDH housekeeping control, hTERT levels in treated cells were expressed as a percent of the untreated control cells. Repression was termed p53-dependent if the percent of the control cells was significantly lower in p53-proficient cells. The results of at least three independent experiments for each isogenic cell line pair are summarized.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Description</th>
<th>Means of p53 inactivation</th>
<th>p53-dependent hTERT repression</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCF7</td>
<td>Breast cancer</td>
<td>siRNA for p53</td>
<td>Yes</td>
</tr>
<tr>
<td>LNCaP</td>
<td>Prostate cancer</td>
<td>GSE596</td>
<td>Yes</td>
</tr>
<tr>
<td>HCT-116</td>
<td>Colon cancer</td>
<td>Gene knockout</td>
<td>No</td>
</tr>
<tr>
<td>HT1080</td>
<td>Fibrosarcoma</td>
<td>GSE596</td>
<td>No</td>
</tr>
<tr>
<td>RKO</td>
<td>Colon cancer</td>
<td>E6</td>
<td>No</td>
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Fig. 2. Repression of the hTERT promoter by wild-type p53. A, shown is a schematic diagram of the luciferase (Luc) reporter plasmids with serial deletions of the hTERT promoter. The arrows designate the transcription initiation site. The lengths of the promoter sequences from the transcription initiation site is shown on the left, and the names of the constructs are indicated on the right. B, H1299 cells were cotransfected with the core hTERT promoter-luciferase (Luc) reporter construct and 10 ng of expression plasmids encoding different p53 mutants (see text). The normalized luciferase activity of the control (con) sample was set to 100%. The results represent the average of at least three independent experiments. The asterisks designate statistically significant repression (p < 0.05; t test). wt, wild-type p53.

Fig. 3. Mapping of p53 domains required for hTERT promoter repression. H1299 cells were cotransfected with the core hTERT promoter-luciferase reporter construct and 10 ng of expression plasmids encoding different p53 mutants (see text). The normalized luciferase activity of the control (con) sample was set to 100%. The results represent the average of at least three independent experiments. The asterisks designate statistically significant repression (p < 0.05; t test). wt, wild-type p53.

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p53 Is Not Associated with the Core hTERT Promoter in Vitro and in Vivo—Since our mapping demonstrated the importance of the intact p53 DNA-binding core domain, we hypothesized that p53 may bind to the hTERT promoter. Examination of the core hTERT promoter sequence revealed a lack of the typical p53 consensus binding sites. However, since this is generally the case for transcriptional repression by p53, we decided to test whether p53 can bind elsewhere to the hTERT promoter. First, we assessed whether p53 can bind to the hTERT promoter in vitro using a Southwestern assay. As shown in Fig. 4A, only background binding of wild-type p53 to the pGL3 plasmid containing the core hTERT promoter could be detected. In contrast, strong binding to the same plasmid containing the p21 promoter (serving as a positive control) was observed. Similar results were obtained with endogenous p53 from doxorubicin-treated MCF7 cells (data not shown).

To examine whether p53 binds to the hTERT promoter in vivo, we performed a chromatin immunoprecipitation experiment. MCF7 cells and their counterparts expressing p53 siRNA were either treated with 0.4 μM doxorubicin for 16 h or left untreated. Following formaldehyde cross-linking and precipitation of the chromatin with anti-p53 polyclonal antibody, the precipitated DNA was subjected to PCR amplification using primers specific to the hTERT promoter or the p53 binding site occurring point mutations in the DNA-binding core domain (R175H and R273H) as well as the inactivating double mutation in the transactivation domain (L22Q/W23S) eliminated p53-mediated repression. Deletion of 51 amino acids (342del), which disrupts the oligomerization domain of p53, also abolished repression. Thus, the integrity of the DNA-binding core domain, the N-terminal transactivation domain, and the C-terminal oligomerization domains is essential for repression. In contrast, deletion of 33 C-terminal amino acids (360del) or the proline-rich domain (62–91del) did not interfere with repression, meaning that these domains are dispensable for this function. The proline-rich domain is important for the recruitment of the Sin3A corepressor and histone deacetylases (32). This mechanism of transcriptional repression by p53 has been demonstrated in the case of stathmin and map4 (33). Thus, these results suggest that recruitment of the Sin3A corepressor by p53 is not involved in hTERT repression. Overall, the pattern of domains essential for hTERT repression is identical to that needed for transactivation by p53.
in the promoter of a known p53 target gene, p21<sup>wtp53</sup>. As shown in Fig. 4B, no amplification of hTERT promoter sequences could be detected, whereas the p21 promoter sequence was easily detected in the same immunoprecipitation product. The specificity of this immunoprecipitation was demonstrated by the lack of a PCR product of the p21 promoter in the no-antibody control and its lower intensity in MCF7-p53i cells. Thus, these results demonstrate that the p53 protein does not associate with the hTERT promoter.

An Atypical E2F Site Is Responsible for Repression of the hTERT Promoter by p53—Since the mutational analysis demonstrated that DNA binding by p53 was required for hTERT repression (Fig. 3), but no direct p53 binding to the core hTERT promoter was apparent, we proposed that p53 must bind elsewhere in the genome to mediate hTERT promoter repression. The complete correlation of the domains necessary for repression with the structural requirements for transactivation by p53 raised the possibility that p53 activates a target gene whose product represses the hTERT promoter. The cell type variability of p53-mediated hTERT repression (Table I) is consistent with this hypothesis and can potentially be explained by the absence of the mediator(s) in some cancer cell lines. We therefore attempted to identify such putative mediator(s) by identification of the cis-regulatory element in the hTERT promoter that is responsible for p53-mediated repression. To this end, we created several deletion and point mutations in the core hTERT promoter-reporter construct as shown in Fig. 5A. The activity of these constructs was studied in MCF7 cells in which the hTERT promoter was repressed by endogenous wild-type p53 (MCF7-vector cells) and compared with that in isogenic cells with knocked-down p53 (MCF7-p53i cells). As shown in Fig. 5B, the activity of the wild-type core promoter was ~3-fold lower in p53-containing cells. Sequential mutation of the Sp1 sites led to a gradual decrease in promoter activity, bringing it to the background level in the mtSp1 construct. These results suggest that Sp1 sites are crucial for the basal activity of the promoter in these cells. In parallel, the difference between control and MCF7-p53i cells progressively decreased, suggesting that when the basal activity is low, the ability of p53 to repress the hTERT promoter is also diminished.

The activity of the construct carrying four mutated Sp1 sites (mt4Sp1) was still repressed by p53 by ~2-fold, but the mutSp1 reporter was almost unaffected by p53, suggesting that the repression maps mainly to the most proximal Sp1 site. However, a point mutation of this site (M6) resulted in only a small decrease in the extent of p53-mediated repression.

Notably, deletion of the 5′-untranslated region (UTR) of the hTERT promoter downstream of the SacII site that contains the most proximal Sp1 site, an E-box, and an overlapping MT-box (20) resulted in a slight increase in promoter activity in p53-proficient cells (MCF7-vector), but a decrease in p53-deficient cells (MCF7-p53i). This deletion almost completely eliminated the p53-mediated repression of hTERT promoter activity. Therefore, we examined the effect of mutation of each of these elements on the extent of p53-mediated repression and found that mutations of the E- or MT-box did not change the degree of repression by p53 (Fig. 5B, M1, M4, and dEM3).

These results suggest that another cis-regulatory element located in the deleted 5′-UTR is involved in the p53-mediated repression exerted by this promoter region. The 5′-UTR contains an atypical E2F-binding site, which was implicated in the activation of hTERT in normal cells by E2F family members (34).

To examine whether repression is mediated via this atypical E2F site, we mutated this site in the core hTERT promoter (Fig. 6A). As demonstrated in Fig. 6B, point mutation of this site (E2F-I) resulted in complete elimination of repression by endogenous p53. In fact, the effect of this mutation was equivalent to the effect of the downstream UTR deletion, as both resulted in elevated activity of the core hTERT promoter in cells containing p53 and in decreased activity in p53-deficient cells. These results suggest that this atypical E2F site upregulates hTERT expression in the absence of active p53 and down-regulates expression when p53 is activated.

E2F and Rb Family Proteins Are Involved in p53-mediated hTERT Repression—To assess more directly the involvement of E2F in the mechanism of hTERT repression, we employed the dominant-negative E2F1 mutant, which can bind DNA, but lacks the transactivation and pRb-binding domains (24). Overexpression of this construct displaces all the endogenous E2F proteins from DNA, resulting in abolishment of both activation and repression by E2F proteins. As demonstrated in Fig. 6C, in the presence of dominant-negative E2F (E2FdTA), p53 was unable to repress core hTERT promoter as demonstrated by the similar activity of the reporter in both p53-proficient and p53-deficient cells.

Next, we tested whether E2F proteins also play such a major role in the p53-dependent regulation of the full-length hTERT promoter. To this end, we performed similar luciferase assays using the 5.9-kb promoter construct. This time, we inactivated p53 transiently by cotransfecting the reporter with the dominant-negative p53-DD peptide. As shown in Fig. 6D, we obtained essentially the same results as for the core promoter construct, indicating that E2F activity is necessary for mediating the p53-mediated repression of the full-length hTERT promoter.

Since the repressive activity of E2F proteins is often mediated by their complexes with pRb family pocket proteins (pRb,
p130, and p107) (35), we also wished to test the involvement of these proteins in hTERT repression. First, we used adenoviral E1A that binds to and inactivates all three pocket proteins (35). As demonstrated in Fig. 6E, the p53-mediated repression of the 5.9-kb hTERT promoter construct was strongly attenuated in the presence of E1A, suggesting involvement of the pRb family in the repression.

However, E1A is known to interact with multiple additional cellular proteins besides pocket proteins. To address more specifically the contribution of Rb family proteins to p53-mediated hTERT repression, we used wild-type human papilloma virus type 16 E7 protein and its deletion mutant that does not bind pocket proteins (E7del21–35). Whereas the wild-type E7 protein significantly hampered p53-mediated repression, E7del21–35 did not.

To further confirm the role of pocket proteins in hTERT repression, we performed similar experiments using a mixture of RNAi targeting individually all three pocket proteins. As demonstrated in Fig. 6E, the p53-mediated repression of the 5.9-kb hTERT promoter construct was strongly attenuated in the presence of E1A, suggesting involvement of the pRb family in the repression.

p21 Mediates p53-dependent hTERT Repression—The results described so far suggest the involvement of a p53 target gene in Rb/E2F-dependent repression of the hTERT promoter. p21cip1/ink4a was found to be the most probable candidate, as it is a p53 transactivation target that links the p53 and Rb/E2F pathways. Induction of p21, an inhibitor of cyclin-dependent kinases, results in accumulation of the hypophosphorylated active form of the Rb family members. These proteins bind to the E2F family transcription factors and turn them from transcriptional activators into repressors (36).

To test the involvement of p21 in hTERT repression, we transiently knocked down endogenous p21 by specific RNAi (p21i) and found that p21 disruption was sufficient to completely eliminate the endogenous p53-mediated repression of the hTERT promoter (Fig. 7A), as its effect was equivalent to the effect of inactivating p53 itself (p53i). The basal activity was measured in MCF7 cells cotransfected with equal amount of mutated p53 siRNA (p53i-mut). Identical results were obtained in LNCaP cells (data not shown).

To confirm the key role of p21 as a mediator of the p53-dependent repression of hTERT, we established a line of MCF7 cells in which p21 was knocked down by stable RNAi (p21i). As a control, MCF7 cells were infected with a virus encoding irrelevant siRNA directed to a region specific for mouse p63 (mp63i) and not found in any human gene. Cells were treated with doxorubicin and analyzed for the expression of the p21 protein and hTERT mRNA. Fig. 7B demonstrates that the level of p21 protein in MCF7-p21i cells following treatment was comparable with the basal level in control MCF7-mp63i cells. As shown in Fig. 7C, the effect of knocking down p21 was equivalent to that of p53, as doxorubicin treatment resulted in only 2-fold repression of hTERT mRNA in both cell lines compared with 5-fold repression in control MCF7-mp63i cells. These results clearly show that p21 is essential for p53-mediated telomerase repression.
p53-mediated Repression of hTERT Requires Histone Deacetylase Activity—The acetylation state of histones has a major influence on transcriptional activity, and recruitment of histone deacetylases is frequently involved in transcriptional repression (37). Hypophosphorylated Rb family members are known to exert active transcriptional repression of genes through their interaction with different corepressor molecules, only a subset of which contains histone deacetylases (38, 39). To examine whether histone deacetylases are involved in the repression of hTERT mRNA, the MCF7 isogenic pair was treated with doxorubicin alone or in combination with the histone deacetylase inhibitor trichostatin A (40). As demonstrated in Fig. 7D, a combination of trichostatin A with doxorubicin strongly attenuated the p53-dependent repression of hTERT expression in MCF7-vector cells. This antagonistic effect was not due to general activation by trichostatin A, as trichostatin A treatment alone slightly repressed hTERT. These results suggest that histone deacetylase activity is involved in the p53-mediated repression of hTERT.

**FIG. 6.** E2F family and pocket proteins are involved in p53-mediated hTERT promoter repression. A, shown is a schematic representation of the core hTERT promoter-luciferase (Luc) reporter construct carrying the E2F-1 mutation. The atypical E2F site, which was mutated in this construct, is boxed, and the mutated nucleotides are marked with asterisks with the substitutions shown above. Other symbols are described in the legend to Fig. 5. B, MCF7 cells stably expressing either the pSuper vector control (MCF7-vector) or p53 siRNA (MCF7-p53i) were transfected with the indicated reporter constructs. Normalized luciferase activity is shown. The data represent one of three independent experiments, each performed in triplicate. dUTR, downstream UTR. C, MCF7-vector and MCF7-p53i cells were cotransfected with the core hTERT promoter-luciferase reporter construct and dominant-negative E2F-dTA or its vector control (con). Normalized luciferase activity is shown. The data represent one of three independent experiments, each performed in triplicate. D, MCF7 cells were cotransfected with the 5.9-kb hTERT promoter-luciferase reporter construct with or without 20 ng of dominant-negative p53-55 expressing plasmid in the presence of dominant-negative E2F-dTA or its vector control. Normalized luciferase activity is shown. The data represent one of three independent experiments, each performed in triplicate. E, MCF7 cells were cotransfected with the 5.9-kb hTERT promoter-luciferase reporter construct with or without 20 ng of p53-55 expressing plasmid in the presence of E1A, E7, mutant E7 defective in pocket protein binding (E7del21–35), or their vector controls. Alternatively, pocket proteins were knocked down by an RNAi mixture (Rb, p107, p130). The bars represent -fold repression as calculated from the ratio of the normalized reporter activity in the presence of p53-DD to the activity in its absence. Each graph represents means ± S.D. of three independent experiments, each performed in triplicate. The ability of different pocket protein-inactivating agents to interfere with p53-mediated repression was estimated by paired t test and found to be statistically significant (marked by asterisks) for E1A (p = 0.0008), E7 (p = 0.002), and the RNAi mixture (p = 0.018), but not for E7del21–35 (p = 0.2).
FIG. 7. p53-mediated repression of hTERT mRNA requires p21 and histone deacetylase activity. A, shown is the luciferase (Luc) activity of the core hTERT promoter in MCF7 cells cotransfected with 600 ng of pSuper vector encoding p53 RNAi (p53i), its mutated control (p53i-mut), or p21 RNAi (p21i). B, MCF7 cells were stably infected with RNAi against p53 (p53i) or a nonspecific RNAi (mp63i) control as described under “Experimental Procedures.” These cells, along with those expressing RNAi against p53 (p53i), were either treated with 0.2 μM doxorubicin (dox) for 16 h or left untreated (con). The Western blot for p21 is shown. Ponceau staining (data not shown) confirmed equal loading. C, the levels of hTERT mRNA in the cells described in B were determined by real-time quantitative RT-PCR. The graph shows -fold repression of hTERT mRNA following doxorubicin treatment after normalization to the GAPDH housekeeping control. D, MCF7-vector and MCF7-p53i cells were treated with 0.4 μM doxorubicin, 100 nM trichostatin A (TSA), or a combination of both for 16 h. The levels of hTERT mRNA were determined by real-time quantitative RT-PCR.

DISCUSSION

Microarray studies suggest that hundreds of genes are repressed by p53 (41, 42). The detailed mechanism of p53 repression was studied for only 20 of these genes. Unlike transcriptional activation by p53, which is mediated through direct p53 binding to defined consensus sites in the regulatory regions of target genes (43), transcriptional repression by p53 is less well understood. The promoters of repressed genes usually do not contain the p53 consensus binding sites. Different mechanisms of p53 transrepression were proposed for different genes. These include interference with the functions of activators either involving p53 binding to DNA or through protein-protein interactions, direct interference with the basal transcription machinery, recruitment of histone deacetylases, and chromatin remodeling (for a review, see Ref. 44).

Our study demonstrates that repression of hTERT expression by endogenous p53 is indirect and is mediated by p21 and the Rb/E2F pathway. An indirect mechanism of transcriptional repression by p53 was suggested previously for other genes. For example, in the case of cdc2, transcriptional repression is also mediated by p21 induction and recruitment of the p130-E2F4 complex to the promoter (45). In addition, the repression of cdk1 by p53 was shown to be mediated by the p21/pRb pathway (46). Notably, the pRb/E2F pathway is not the only one that can mediate gene repression following activation of p53 and p21. A recent study shows that inhibition of cdk2 by p21 leads to impaired DNA binding activity of the NF-Y transcription factor (47). This in turn causes down-regulation of cell cycle regulatory genes such as cyclin B1 and cdc2, leading to G2 arrest (48).

Based on all our results, we propose a model in which p53 mediates the repression of hTERT expression through p21 induction. Activation of the Rb family is at least partially responsible for the recruitment of a histone deacetylase-containing repressive complex to the hTERT promoter through an atypical E2F site. However, since the repression was completely dependent on p21 induction and E2F activity but only partially pocket protein-dependent, it is possible that p21 can exert E2F-dependent repression through an additional “pocket-independent” arm. We are currently investigating this intriguing possibility.

The biological significance of telomerase repression by p53 remains to be determined. When human fibroblasts are transfected with SV40 large T antigen, which binds to and inactivates p53 and pRb, telomerase activation does not occur until further genetic alterations are acquired (49). Similar results were obtained with normal human breast epithelial cells in which p53 mutated at codon 143, 175, 248, or 273 was overexpressed (50). In an additional study, a combination of E1A, MDM2, and Ras did not induce hTERT expression, although it caused full transformation of normal human cells; telomerase reactivation occurred only later during ex vivo culture of these engineered tumor cells (51). Taken together, these results suggest that inactivation of the p53 and Rb pathways, even together with overexpression of active Ras, is not sufficient for the immediate robust hTERT activation that could be detected in an asynchronous cell population. Presumably, p53 inactivation contributes to hTERT activation indirectly by causing the genomic instability that is observed in a crisis. As these studies have shown, it is during this period that the oncogenic changes leading to robust hTERT up-regulation occur. Recently, this idea was confirmed in human mammary epithelial cells, where loss of p53 function accelerated acquisition of telomerase activity (52).

Moreover, it is possible that the repression of hTERT by p53 is important for the recently reported strict cell cycle-dependent control of hTERT expression in normal cells (53). We speculate that deregulation of this strict control, which was found in cancer cells (53), is associated with inactivation of the p53/p21/pRb pathway inherent to almost all tumors.

In complete agreement with our conclusions are the results of a recent study showing that dynamic assembly of the E2F-pocket protein-histone deacetylase complex plays a central role in the regulation of hTERT in a variety of proliferative conditions (e.g. normal cycling, senescent, and tumor cells) (54). Notably, the E2F site in the hTERT promoter that was found to
be responsible for the E2F-pocket protein-histone deacetylase regulation of hTERT (54) was mapped by us as responsible for p53-mediated repression.

Therefore, hTERT seems to belong to a large group of Rb/E2F-regulated genes, the function of which is essential for cell cycle progression. Several members of this group were reported to be regulated genes, the function of which is essential for cell cycle progression. Several members of this group were reported to be regulated genes, the function of which is essential for cell cycle progression. Several members of this group were reported to be regulated genes, the function of which is essential for cell cycle progression. Several members of this group were reported to be regulated genes, the function of which is essential for cell cycle progression.

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