Expression of an IFN-Inducible Cellular Senescence Gene, IFI16, Is Up-Regulated by p53

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Abstract

IFN-inducible IFI16 protein (encoded by IFI16 gene at 1q23.1) is the human member of the IFN-inducible structurally related p200 family proteins. Increased expression of the IFI16 protein, a positive modulator of p53-mediated transcription, in normal old human diploid fibroblasts (HDF) is associated with cellular senescence-mediated cell growth arrest. However, the underlying mechanisms that contribute to transcriptional activation of the IFI16 gene in old HDFs remain to be elucidated. Here, we reported that functional activation of p53 in normal young HDFs and p53-null Saos2 cell line resulted in transcriptional activation of the IFI16 gene. We identified a potential p53 DNA-binding site (indicated as IFI16-p53-BS) in the 5′-regulatory region of the IFI16 gene. Importantly, p53 bound to IFI16-p53-BS in a sequence-specific manner in gel-mobility shift assays. Furthermore, p53 associated with the 5′-regulatory region of the IFI16 gene in chromatin immunoprecipitation assays. Interestingly, p53 associated with the regulatory region of the IFI16 gene only on treatment of cells with DNA-damaging agents or in the old, but not in the young, HDFs. Importantly, our promoter-reporter assays, which were coupled with site-directed mutagenesis of IFI16-p53-BS, showed that p53 activates transcription of the IFI16 gene in HDFs through the p53 DNA-binding site. Together, our observations provide support for the idea that up-regulation of IFI16 expression by p53 and functional interactions between IFI16 protein and p53 contribute to cellular senescence.

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Introduction

Replicative or cellular senescence-associated permanent cell growth arrest of normal human cells serves as a major barrier to tumor formation (1-7). The cell growth arrest limits the ability of normal cells to proliferate and preserves genomic integrity by preventing cells with short telomeres from entering the cell cycle (2, 3, 7).

p53 protein level increases in certain normal old human diploid fibroblasts (HDF), such as IMR90 and MRC5, during replicative senescence (8-12). Likewise, the p53 sequence-specific DNA-binding activity and transcriptional activity increase during replicative senescence (9). Moreover, functional inactivation of p53 using viral oncoproteins, such as SV40 large T antigen, or knockdown of p53 expression allows cells to bypass the onset of replicative senescence, resulting in an extension of proliferation potential of cells (13). These studies (8-13) have established a role for p53 in cellular senescence.

Functional inactivation of p53 by genetic alterations contributes to >50% human cancers (14-19). p53 is a transcription factor, which is activated in cells in response to certain stimuli, such as DNA damage, hypoxia, oxidative stress, or other cellular stress (16-18). Moreover, telomere shortening in normal human cells triggers cellular senescence through activation of ataxia-telangiectasia mutated kinase, which phosphorylates p53 on Ser15, resulting in activation of p53 (10-12). The activated p53 binds to its DNA-binding consensus sequence that is present in its target genes (12-15). The binding of p53 to its target genes is known to result in transcriptional activation of genes, such as p21 and Gadd45, or transcriptional repression of genes, such Bcl2 and MAP4 (12, 13). The p53-regulated genes encode proteins that mediate tumor suppressor function of p53 in normal human cells by inducing cell growth arrest, apoptosis, or senescence (14-17). The p53-mediated up-regulation of p21 expression in normal old human old fibroblasts is known to contribute to cellular senescence-associated cell growth arrest (11). In contrast, knockout of the p21 gene in human cells increases the proliferation potential of cells (20).

IFI16 gene is a member of IFN-inducible IFI200 gene family (21). The family includes mouse (e.g., Ifi202a, Ifi202b, Ifi203, and Ifi204) and human (e.g., IFI16, MNDA, AIM2, and IFIX) genes that encode structurally related proteins (termed p200 family proteins). Increased expression of the p200 family proteins, such as p202 and p204 (encoded by the Ifi202 and Ifi204 genes, respectively), inhibits cell cycle progression (22, 23). Interestingly, the p202 protein inhibits cell cycle progression in part through up-regulation of p21 expression, by decreasing phosphorylation of Rb, and through inhibition of E2F-mediated transcription of growth-promoting genes (22, 23). Consistent with a role for p200 family proteins in cell cycle regulation, we have noted (21) that increased expression of the...
IFI16 protein in old (versus young) populations of normal human prostate epithelial cells (24) and normal HDFs (25) is associated with cellular senescence-induced cell growth arrest. Moreover, immortalization of normal human fibroblasts with hTERT or SV40 large T antigen is associated with down-regulation of the IFI16 mRNA and protein (25). Furthermore, knockdown of IFI16 expression in HDFs results in down-regulation of p21CIP1 protein levels and increases in the proliferation potential (25). Consistent with a role for IFI16 protein in cellular senescence-associated cell growth arrest, we noted that IFI16 protein up-regulates the expression of p21CIP1 and inhibits E2F-mediated transcription (24, 25). Interestingly, increased coexpression of the IFI16 and p53 proteins in MCF-7 cells enhanced the transcription of the known p53 target genes, such as p21, Hdm2, and Bax (26). Importantly, knockdown of IFI16 expression in MCF-7 cells results in decreased phosphorylation of p53 on Ser15 following ionizing radiation treatment and decreased p53-mediated apoptosis (26). Additionally, the IFI16 protein binds to p53 (27) and increased expression of the IFI16 protein in cells potentiates p53-mediated transcription of p21-luc-reporter (27). These observations (25-27) provide support for the idea that increased levels of the IFI16 protein in old cells, by potentiating p53-mediated transcription of cell cycle inhibitory genes, such as p21, contribute to senescence-associated permanent cell growth arrest. Although these observations suggest a role for IFI16 protein in p53-mediated cellular senescence, signaling pathways that contribute to transcriptional activation of the IFI16 gene during cellular senescence remain to be elucidated.

Because expression of the IFI16 gene is up-regulated in normal old (versus young) human WI-38 fibroblasts (25) and p53 is activated during the onset of cellular senescence (10-12), we explored whether activation of p53 protein in cells could regulate the IFI16 expression. We report that activation of p53 in normal human fibroblasts stimulates the transcription of the IFI16 gene. These observations provide support for the idea that p53-mediated up-regulation of IFI16 expression in old cells and the functional interactions between IFI16 and p53 proteins contribute to cellular senescence-associated cell growth arrest.

**Results**

Activation of p53 in Human Old Fibroblasts Increases IFI16 Expression Levels

IFI16 protein and mRNA levels increase in normal human fibroblasts when they approach cellular senescence or when cells become old (65-75% cells in culture morphologically appear large and flat and stain positive for acidic senescence-associated β-galactosidase; ref. 25). Because cellular senescence in HDFs is associated with functional activation of p53 through phosphorylation (10, 11), we tested whether the functional activation of p53 in old WI-38 fibroblasts is associated with increases in IFI16 protein levels. As shown in Fig. 1A, continuous culture of young [26 population doublings (PD)] WI-38 cells under culture conditions resulted in accumulation of morphologically senescent cells in culture after 62 PDs. In addition, as described earlier (25), in these cultures, about 65% to 75% of cells also stained positive for...
Inhibition of p53 Function Decreases IFI16 Protein Levels

Immortalization of WI-38 fibroblasts with SV40 large T antigen resulted in measurable decreases in IFI16 protein expression levels compared with normal WI-38 cells (25). Because SV40 large T antigen binds to p53 and inactivates p53-mediated functions (13), we tested whether treatment of cells with pifithrin-α, an inhibitor of p53-mediated functions, has any effect on IFI16 expression levels. As shown in Fig. 3, treatment of cells with pifithrin-α (30 μmol/L for 24 hours) resulted in measurable decreases in p21 and IFI16 protein levels. This observation revealed that basal expression levels of both p21 and IFI16 proteins in WI-38 cells depend on functional p53 protein.

Restoration of p53 Function in p53-Null Cells Increases IFI16 Protein Levels

To further examine the role of p53 in the regulation of IFI16 expression, we chose to use a well-characterized human osteosarcoma Saos2 cell system (Saos2 cells are null for p53), which expresses a temperature-sensitive mutant (Val138) of p53 with either Pro or Arg amino acid residue in position 72 (29). Incubation of cells that express the temperature-sensitive mutant of p53 with Pro (SaosPro72) or Arg (SaosArg72) at 32°C is known (29) to change the conformation of p53 to an active conformation, resulting in transcriptional activation of the p53 target genes. As shown in Fig. 4A, incubation of SaosPro72 or SaosArg72 at 32°C for 24 hours resulted in up-regulation of p21CIP1, a transcriptional target of p53 (30). Interestingly, the incubation of cells at 32°C also resulted in increases in levels of the IFI16 protein (Fig. 4A, compare lane 2 with lane 3). Because increases in levels of the IFI16 protein were relatively more in SaosArg72 cells than in SaosPro72 cells, we further compared expression levels of the IFI16 protein in SaosPro72 cells after their incubation at 39°C and 32°C (for 24 or 48 hours). Consistent with our previous observations (25), incubation of cells at 32°C for 24 or 48 hours resulted in up-regulation of p21CIP1 and IFI16 protein levels (Fig. 4B, compare lanes 2 and 3 with lane 1). To rule out the possibility that incubation of parental Saos2 cells at reduced (32°C) temperature could account for increases in IFI16 protein.

<table>
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<tr>
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<tr>
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<tr>
<td>IFI16</td>
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<td>Actin</td>
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**FIGURE 2.** Activation of p53 in young proliferating WI-38 fibroblasts by bleomycin treatment results in increases in IFI16 protein levels. Young proliferating WI-38 fibroblasts were either left untreated (lane 1) or treated with bleomycin (3.0 × 10³ units/mL) for the indicated time. Total cell lysates were prepared and analyzed by immunoblotting using antibodies specific to the indicated proteins.

<table>
<thead>
<tr>
<th>Condition</th>
<th>p53</th>
<th>p-p53</th>
<th>IFI16</th>
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**FIGURE 3.** Treatment of WI-38 cells (PD 28) with an inhibitor of p53 function decreases p21 and IFI16 protein levels. WI-38 cells were treated with either DMSO (lane 1) or pifithrin-α (PFT-α; 30 μmol/L; lane 2) for 24 h. After the treatment, total cell lysates were analyzed by immunoblotting using antibodies specific to the indicated proteins.

acidic β-galactosidase (data not shown). Therefore, we compared activation of p53 protein between young and old cells. As shown in Fig. 1B, we could detect phosphorylation of p53 on Ser15 and a moderate (50-60%) increases in p53 protein levels in old cells (compare lane 2 with lane 1). Moreover, we noted that levels of the IFI16 protein in extracts from old WI-38 cells were relatively higher than young proliferating cells (Fig. 1B, compare lane 2 with lane 1). Furthermore, we also noted increases in levels of cyclin-dependent kinase inhibitors, such as p21CIP1 and p16, and pRb in extracts from old WI-38 cells. Additionally, consistent with our previous observations (25), we noted decreases in levels of Id1 protein in old WI-38 cells. Similarly, we also found that levels of the IFI16 protein were measurably higher in old versus young IMR90 HDFs and the increase in the IFI16 protein levels was associated with increases in p53 protein levels (data not shown). Consistent with increases in IFI16 protein levels in old WI-38 and IMR90 HDFs, we noted that levels of both IFI16 and p21 mRNA levels were relatively higher in old than young WI-38 cells (Fig. 1C).

To further examine whether the activation of p53 in old WI-38 cells results in increases in IFI16 protein levels, we treated young (25 PDs; ref. 25) proliferating WI-38 fibroblasts with bleomycin, a DNA-damaging agent that is known (28) to cause single- and double-stranded breaks. As shown in Fig. 2, treatment of young proliferating WI-38 fibroblasts with bleomycin (3,000 units/mL) for increasing lengths of time, which resulted in activation of p53 (as determined by increases in phosphorylation of Ser15 in p53) within 5 hours of treatment (compare lane 2 with lane 1), resulted in increases in p21CIP1 protein levels. Interestingly, an increase in IFI16 protein levels was also evident after 5 hours of treatment; however, the maximum increase in IFI16 protein levels was noted after 17 hours of treatment. Similarly, treatment of young proliferating WI-38 fibroblasts with increasing (20-80 μmol/L) concentrations of etoposide for 24 hours, which resulted in phosphorylation of p53 on Ser15 and stabilization of p53, also resulted in increases in p21CIP1 and IFI16 protein levels (data not shown). Together, these observations suggested that functional activation of p53 in young WI-38 cells by DNA-damaging agents, such as bleomycin or etoposide, is associated with increases in IFI16 protein levels.
levels (independent of p53 expression), we also compared the IFI16 protein levels between the Saos2 cells that were incubated at 39°C or 32°C. We found no difference between IFI16 protein levels in extracts from cells incubated at these two different temperatures (Fig. 4C). Together, these observations suggested that restoration of p53 function in Saos2 cells increases IFI16 protein levels.

**Functional p53 Activates Transcription of the IFI16 Gene**

To determine whether the activation of p53 in cells increases levels of the IFI16 protein through transcriptional mechanisms, we transfected SaosPro72 or SaosArg72 cells with IFI16-luc-reporter plasmid in which transcription of the luciferase reporter gene is driven by the 5' regulatory region (1.68 kb) of the IFI16 gene (31). As shown in Fig. 5, incubation of either SaosPro72 or SaosArg72 cells at 32°C for 48 hours measurably stimulated the activity of the reporter more than 50% to 80% in two independent experiments. Similarly, incubation of these cells at 32°C also resulted in stimulation of p21-luc-reporter activity, indicating that the incubation of cells at 32°C activated p53 and this activation resulted in transcriptional activation of p53 target genes, such as IFI16 and p21.

**p53 Associates with the 5'-Regulatory Region of the IFI16 Gene That Contains a Potential DNA-Binding Site**

The above observations prompted us to search for a potential p53 DNA-binding site(s) in the 5'-regulatory region of the IFI16 gene. Our search identified a single putative p53 DNA-binding site (designated as IFI16-p53-BS) within the first exon of the IFI16 gene (Fig. 6A; Table 1). After identification of potential IFI16-p53-BS in the IFI16 gene, we sought to determine whether p53 could associate with this site in response to activation of p53 in cells. For this purpose, we did chromatin immunoprecipitation (ChIP) assays using either control...
antibodies or antibodies to p53 and immunoprecipitated chromatin was amplified using a pair of primers flanking the IFI16-p53-BS in the 5' regulatory region of the IFI16 gene or a primer pair that flanked the p53 DNA-binding site in the p21 gene. As shown in Fig. 6B, we could detect association of p53 with the 5'-regulatory region of the IFI16 gene in old, but not young (compare lane 8 with lane 7), WI-38 cells in ChIP assays. Moreover, we also detected an association of p53 with the regulatory region of the IFI16 gene in young WI-38 cells that were treated with doxorubicin (compare lane 9 with lane 7), a DNA-damaging agent (32). As a positive control, we also did ChIPs for the association of p53 in the 5'-regulatory region of the p21 gene in the same experiments. Together, these observations indicated that on activation of p53 in WI-38 cells, the activated p53 associates with the 5'-regulatory region of the IFI16 gene, which contains a potential p53 DNA-binding site.

p53 Binds to IFI16-p53-BS in Gel-Mobility Shift Assays

Association of p53 to the 5'-regulatory region of the IFI16 gene in cells in ChIP assays prompted us to test whether p53 binds to IFI16-p53-BS in a sequence-specific manner. For this purpose, we analyzed extracts from untreated or doxorubicin-treated WI-38 cells (passage 6B) by gel-mobility shift assays using digoxigenin (DIG)-labeled oligonucleotide containing either the wild-type IFI16-p53-BS or the p53 DNA-binding consensus sequence (p53-con). As shown in Fig. 7A, p53 in extracts bound to the labeled oligonucleotide containing either IFI16-p53-BS or p53-con sequence from untreated cells. This is because we chose WI-38 cells at passage 36 when cell cultures were neither young nor old, resulting in activation of p53 in some WI-38 cells. Moreover, treatment of cells with increasing concentration of doxorubicin, which resulted in phosphorylation of p53 on Ser^15^ and up-regulation of p21(CIP^2^ (Fig. 7B), measurably increased the binding of p53 to both oligonucleotides. Furthermore, mutations in the IFI16-p53-BS measurably decreased the binding of p53 from both control and doxorubicin-treated WI-38 cells (Fig. 7C), indicating that p53 binds to IFI16-p53-BS in a sequence-specific manner. To further examine the specificity of p53 DNA binding to IFI16-p53-BS, we did competition experiments using unlabeled oligonucleotide containing either the wild-type IFI16-p53-BS or the p53-con sequence. Consistent with our previous observations (Fig. 7C), p53 from control or doxorubicin-treated cells bound to the labeled wild-type IFI16-p53-BS (Fig. 7D). However, preincubation of nuclear extracts with unlabeled oligonucleotide containing either IFI16-p53-BS or the p53-con sequence measurably decreased the binding of the labeled oligonucleotide (containing IFI16-p53-BS) to p53. These observations clearly showed that p53 binds to IFI16-p53-BS in a sequence-specific manner.

![Diagram of p53 DNA-binding site](http://example.com/diagram)

**FIGURE 6.** p53 associates with the 5'-regulatory region of the IFI16 gene that contains a potential p53 DNA-binding site in vivo. A. The location and the sequence of a putative p53 DNA-binding site in the IFI16 gene. The location of forward and backward primers that were used to amplify immunoprecipitated chromatin DNA for ChIP assays is also shown. B. Young proliferating (PD 14; lanes 1, 4, and 7), old (PD 60; lanes 2, 5, and 8), or young proliferating cells that were treated with doxorubicin (200 ng/mL for 24 h) were cross-linked and chromatin from cells was immunoprecipitated with either isotype antibodies (lanes 4-6) or anti-p53 antibodies (lanes 7-9). DNA fragments extracted from the immunoprecipitates were PCR amplified with the primer sets that encompass putative p53-binding site of the IFI16 gene.

<p>| Table 1. A Comparison of p53 DNA-Binding Site Sequence That Is Present in the IFI16 Gene with Other p53 DNA-Binding Sites Present in Other Known p53 Target Genes |</p>
<table>
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<th>Gene of Origin</th>
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<tr>
<td>Consensus</td>
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<tr>
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<tr>
<td>Mdm-2/a</td>
<td>CGTCAAGTTGGACACGCC</td>
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<tr>
<td>Gadd45</td>
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<tr>
<td>IFI16</td>
<td>GAGAAGCCACGCACAGTCA</td>
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**NOTE:** Bold nucleotides indicate a variation in the nucleotide from the p53 DNA-binding consensus sequence.
p53 Transcriptionally Activates the IFI16 Gene in IFI16-p53-BS–Dependent Manner

Association of p53 with the 5’-regulatory region of the IFI16 gene in ChIP assays (Fig. 6B), binding of p53 to IFI16-p53-BS in gel-mobility shift assays (Fig. 7), and stimulation of the activity of IFI16-luc-reporter by functional p53 in Saos2 cells (Fig. 5) provided support to the idea that p53 activates the transcription of the IFI16 gene through IFI16-p53-BS. To test this possibility, we mutated IFI16-p53-BS in IFI16-luc-reporter to a low-affinity DNA-binding site by site-directed mutagenesis and compared the activity of the wild-type IFI16-luc-reporter with the mutant IFI16-luc-reporter in WI-38 cells without or after doxorubicin treatment. As shown in Fig. 8, treatment of cells with doxorubicin resulted in stimulation of the activity of wild-type reporter about 50% to 70%. However, the treatment did not result in stimulation of the activity of the mutant reporter irrespective of the concentration of doxorubicin treatment. Because the treatment of cells with doxorubicin stimulated the activity of p21-luc-reporter robustly in the same experiments, our observations indicated that the activation of p53 in WI-38 cells stimulates the transcription of the IFI16 gene in IFI16-p53-BS–dependent manner (Fig. 8).

Discussion

p53 protein levels increase in HDFs, such as IMR90 and MRC5, during replicative senescence (8-11). Moreover, p53 sequence-specific DNA-binding activity and transcriptional activity also increase during replicative senescence (9, 12). These studies (8-12) have suggested a role for p53 in the onset and maintenance of cellular senescence. Consistent with this idea, the p53-mediated induction of p21 and Gadd45 genes in normal human cells is known (13, 16, 17) to play a role in cell growth arrest. However, the number of p53 target genes whose expression is induced during cellular senescence in HDFs remains rather limited. Therefore, our observations that p53 activates the expression of IFI16, a candidate cellular senescence gene, in response to certain DNA damage signals in normal human fibroblasts are important.
result in up-regulation of the IFI16 mRNA and protein (21). Because steady-state levels of the IFI16 mRNA increase in older populations of HDFs (25), it seems likely that the increases in IFI16 protein levels in old HDFs are, in part, due to the transcriptional activation of the IFI16 gene. Because activation of p53 in normal old human fibroblasts is known (10, 11) to activate the transcription of p21 gene that encodes a negative regulator of cyclin-dependent kinases (30), we explored whether p53 could also regulate the expression of the IFI16 gene that encodes a candidate cellular senescence gene. Our observations revealed that (a) activation of p53 in old WI-38 fibroblasts was associated with increases in IFI16 protein and mRNA levels (Fig. 1), (b) activation of p53 in WI-38 cells in response to DNA-damaging agents (e.g., bleomycin or etoposide) was associated with increases in IFI16 protein levels (Fig. 2), (c) restoration of p53 function in Saos2 cells up-regulated IFI16 protein levels (Fig. 4) and stimulated the activity of IFI16-luc-reporter (Fig. 5), (d) p53 associated with the 5′-regulatory region of the IFI16 gene containing a potential p53 DNA-binding site (IFI16-p53-BS) in chromatin precipitation assays (Fig. 6), (e) the endogenous p53 bound to the IFI16-p53-BS in sequence-specific manner in gel-mobility shift assays (Fig. 7), and (f) p53 stimulated the activity of IFI16-luc-reporter in IFI16-p53-BS—dependent manner (Fig. 8). Together, our observations provide evidences that, on activation in response to certain DNA damage signals, p53 activates the transcription of the IFI16 gene in normal human fibroblasts.

Studies have provided evidence that increased levels of the IFI16 protein in cells potentiate p53-mediated transcription of target genes (25-27). Moreover, it has been shown that IFI16 protein is needed for p53-mediated apoptosis in certain cells following ionizing radiation–induced apoptosis (26). Consistent with a role for IFI16 protein in potentiating p53-mediated functions, inactivation of p53 with human papillomavirus 16–encoded E6 protein in human umbilical vein endothelial cells impaired the ability of the IFI16 protein to inhibit the in vitro angiogenic activity and cell cycle progression (33). Therefore, our observations that expression of IFI16 in HDFs depends on p53 function are important.

Mouse embryonic fibroblasts, which are null for IFN-regulatory factor-1 (IRF-1), are deficient in their ability to undergo DNA damage–induced cell cycle arrest (33). A similar

![Graph](attachment:graph.png)

**FIGURE 8.** p53 stimulates transcription of the IFI16 gene in IFI16-p53-BS—dependent manner. A. IFI16 promoter-driven luciferase reporters containing the wild-type or mutated IFI16-p53-BS. B. WI-38 cells (in 24-well plate) were transfected with the wild-type IFI16 luciferase reporter plasmid (20 ng/well) or the mutant IFI16 luciferase reporter plasmid plus pRL-TK luciferase reporter plasmid (1 ng/well) as an internal control. Twenty-four hours after transfection, cells were treated with DMSO alone (control) or the indicated concentrations of doxorubicin. Dual-luciferase assays were done after 24-h treatment of doxorubicin. C. WI-38 cells (in 24-well plate) were transfected with p21 luciferase reporter plasmid (20 ng/well) plus pRL-TK luciferase reporter plasmid (1 ng/well) as an internal control. Twenty-four hours after transfections, cells were treated with various concentrations of doxorubicin, and dual-luciferase assays were done. In experiments shown in B and C, the relative luciferase activity (RLU) was expressed as the ratio between the firefly luciferase/Renilla luciferase/mg of proteins. Columns, mean (n = 3); bars, SD. Data are expressed considering 1.0 as the relative luciferase activity of cells treated with DMSO alone.
IFN-activatable genes is up-regulated during the onset of senescence, studies have revealed that expression of certain human cells. Consistent with a role for IRF-1 in cellular response to DNA damage signal that is generated in normal old cells with the IRF-1 to activate transcription of certain target genes in light of these observations, it is conceivable that p53 cooperates with IRF-1 to activate transcription of certain target genes in response to DNA damage signal that is generated in normal old human cells. This assumption is based on previous studies that demonstrated a role for IRF-1 in cellular senescence, and the observation that expression of certain genes is up-regulated during the onset of cellular senescence in normal human prostate epithelial cells (35, 36). Moreover, inhibition of DNA methylation by 5-aza-2'-deoxycytidine, an inhibitor of DNA methyltransferase, in immortalized fibroblasts results in up-regulation of IRF-1, activation of IFN-activatable genes, and a senescence-like phenotype (37). Therefore, further work will be needed to determine whether the transcription factor IRF-1 cooperates with p53 in human cells to activate the transcription of the IFI16 gene.

The IFI16 protein binds to the COOH terminus (amino acids 362-393) of p53 (27) and overexpression of the IFI16 protein in cells increases p53 protein levels and stimulates the p53-mediated transcription (25, 38). Conversely, knockdown of IFI16 expression results in down-regulation of p53 levels (25). Notably, overexpression of IFI16 in immortal cell lines up-regulates the p21 expression and inhibits the E2F-mediated transcription (24). Similarly, overexpression of IFI16 in primary human umbilical vein endothelial cells up-regulates p53 and p21 expression (33). Furthermore, knockdown of IFI16 expression in WI-38 cells results in down-regulation of p21 expression and decreases in the activity of the p21-luc-reporter (25). Therefore, our observations described here support the idea that increased levels of the IFI16 protein in normal old human cells contribute to senescence-associated cell growth arrest, in part, through the p53/p21 pathway and up-regulation of IFI16 expression by p53 in normal old human cells that are associated with higher levels of the IFI16 protein. Because the Pro/Pro genotype has a 41% increased survival of individuals (despite a 2.54-fold increased proportional mortality from cancer) compared with the individuals with the Arg/Arg genotype (40) and steady-state levels of the IFI16 mRNA vary among individuals (21), further work will be needed to determine whether a particular genotype of p53 is associated with cellular expression levels of the IFI16 protein. Differences in the expression levels of the IFI16 protein among individuals could account for certain aging-dependent diseases, including certain human cancers (21).

In summary, our observations will serve as a basis to understand how the loss of p53 and/or IFI16 function in cells contributes to defects in cellular senescence-associated cell growth arrest, resulting in certain aging-dependent human cancers.

Materials and Methods

Cell Cultures and Reagents

Young HDFs WI-38 (at 23 PDs), IMR90 (at 26 PDs), and BJ (at 23 PDs) were purchased from the American Type Culture Collection and maintained in culture (CO₂, 5%; oxygen, 20%) as suggested by the supplier. Cell cultures were regularly split 1:4 on approaching confluence. Thus, each cell passage was equivalent to ~2 cell PDs. When 65% to 75% of cells in culture morphologically appeared large and stained positive for senescence-associated cell growth arrest, resulting in certain aging-dependent human cancers.

Human osteosarcoma Saos2 cells (29) that stably expressed a temperature-sensitive mutant (Val₁³⁸) of human p53 with either Arg²⁷ (cells indicated as Saos²⁷) or Pro²⁷ (cells indicated as Saos²⁷) were generously provided by Dr. Paul Murphy (Fox Chase Cancer Center, Philadelphia, PA). Young HDF WI-38 cells were purchased from the American Type Culture Collection. Both Saos2 and WI-38 cells were maintained in DMEM culture medium (Invitrogen).
supplemented with 10% fetal bovine serum and 5% CO₂ at 37°C. For experimental purpose, Saos_2 and Saos_3 cells were incubated either at 39°C (favoring p53 mutant conformation) or at 32°C (favoring p53 wild-type conformation; ref. 29). Chemicals, other than indicated, were purchased from Sigma.

Plasmids and Expression Vectors

The wild-type IFI16-luc-reporter plasmid, which contains the promoter region (1.677 kb; 1,467 bp upstream of the transcriptional start site and 210 bp downstream of the start site) of the IFI16 gene, was constructed (31) using pGL3-luc basic vector (Promega). The IFI16-luc-reporter plasmid with mutant p53 DNA-binding site in the IFI16 gene was generated using the QuickChange Site-Directed Mutagenesis kit (Stratagene) according to the manufacturer’s instructions. Briefly, two oligonucleotide primers containing the desired mutations for the p53 DNA-binding site, each complementary to the opposite DNA strand of the parental plasmid vector, were extended using the wild-type IFI16-luc-reporter plasmid as a template during temperature cycling by PfuTurbo DNA polymerase. The amplification was done for 18 cycles using the following conditions: 95°C for 30 s, 55°C for 1 min, and 68°C for 7 min. The mutated clones were selected and amplified and the mutations were verified by DNA sequencing. The following oligonucleotide primers were used to generate mutations in IFI16-p53-BS in the IFI16-luc-reporter plasmid: 5’-CAATAG-CAGAATAGGATCAAACCAGGTATCTCAGCTAAC-TAAC-3’ and 5’-CTTAGGATGGATGATCGGCTTAC-TCTCTATCCGTAATG-3’ (the mutated nucleotides are underlined).

Transient Transfection and Dual-Luciferase Assays

All transient transfection assays were done using FuGene6 transfection reagent (Roche Diagnostics) according to the manufacturer’s instructions. In brief, subconfluent cells were cotransfected with desired reporter plasmid (IFI16-luc or p21-luc) along with pRL-TK plasmid as an internal control. Forty-eight hours after transfections of cells, firefly luciferase and Renilla luciferase activities were assayed using Dual-Luciferase Reporter Assay kit (Promega). Relative luciferase activity was expressed as the ratio of the firefly luciferase/Renilla luciferase/mg protein. Student’s t test for paired samples was used to determine statistical significance of the reporter activity data. Differences were considered statistically significant at P ≤ 0.05.

Real-time PCR

Total RNA was isolated from young and old WI-38 fibroblasts with Trizol reagent (Invitrogen). cDNA synthesis was done using primers with SuperScript First-Strand Synthesis System for reverse transcription-PCR (Invitrogen). Quantitative real-time Taqman PCR technology (Applied Biosystems) was used to determine the differential gene expression between the young and old HDFs. The PCR cycling program consisted of denaturing at 95°C for 10 min and 40 cycles at 95°C for 15 s and annealing and elongation at 60°C for 1 min. The Taqman assays for IFI16 (assay Id Hs00194216_m1), p21 (assay Id Hs00355782_m1), and the endogenous control β-actin (assay Id Hs99999903_ml) were purchased from Applied Biosystems and used as suggested by the supplier.

ChIP Assays

ChIP assays were done essentially using a protocol suggested by Upstate Biotechnology with minor modifications. In brief, WI-38 cells (0.5 × 10^8) were fixed with 1% formaldehyde at room temperature for 10 min. The cross-linking was quenched with 0.1 mol/L glycine for 5 min at room temperature. Cells were washed twice with PBS and lysed for 30 min at 4°C in a lysis buffer. The cell lysates were sonicated into chromatin fragments with the average length of approximately 200 to 800 bp, as assessed by agarose gel electrophoresis. The samples were precleared with salmon sperm DNA/protein A-agarose (Upstate Biotechnology) for 2 h at 4°C on a rocking platform, and the specific antibodies (10 µg) were added, and the samples were rocked overnight at 4°C. Immunoprecipitates were captured with salmon sperm DNA/protein A-agarose beads for 2 h on a rocking platform. PCR was done using a pair of primers (forward, 5’-AATAGCAGAATAGGAGCAAG-3’; reverse, 5’-GACCCGGATGAAATACCAATCTG-3’) that encompasses the putative p53 DNA-binding site in the 5’-regulatory region of the IFI16 gene. As a positive control, the promoter region of the p21 gene containing the p53 DNA-binding site was amplified with the following primer set: forward, 5’-GTTGCCCTCTATGTTTCCTTCTG-3’; reverse, 5’-CTGAAAAACGAGCCACCAAG-3’.

Immunoblotting

Cell lysates were prepared in radioimmunoprecipitation assay buffer [50 mmol/L Tris-HCl (pH 7.5), 150 mmol/L NaCl, 1% NP40, 0.5% deoxycholate, 0.1% SDS, including 1× protease inhibitor (Roche Diagnostics)]. Equal amounts of protein (20 µg) were resolved by SDS-PAGE and transferred to polyvinylidene difluoride membranes. Membranes were probed overnight at 4°C with the following primary antibodies: anti-IFI16 antibody (Santa Cruz Biotechnology) at 1:200 dilution, anti-p21 antibody (Santa Cruz Biotechnology) at 1:200 dilution, anti-p53 antibody (DO1, Santa Cruz Biotechnology) at 1:500 dilution, and anti-β-actin (Cell Signaling) at 1:1,000 dilution. Protein-antibody complexes were detected with horseradish-conjugated secondary antibodies by using an enhanced chemiluminescence system (Amersham Biosciences).

Electrophoretic Gel-Mobility Shift Assays

Sequence-specific DNA-protein interactions were subjected to electrophoretic gel-mobility shift assay analysis by using DIG Gel-Shift kit (Roche Diagnostics) according to the manufacturer’s instructions. Briefly, single-strand DNA oligonucleotides were annealed and labeled with DIG-11-ddUTP and used as probes. Equal amount of cell extracts was then incubated with DIG-labeled probes in the presence or absence of 40-fold excess of an unlabeled oligonucleotide at 20°C for 20 min to allow DNA/protein complexes to form. The DIG-labeled DNA/protein complexes were resolved on 6% DNA retardation gel (Invitrogen) and then transferred to a nylon positively charge membrane (Roche Diagnostics). The DIG-labeled DNA/protein complexes were then visualized by an
enzyme immunoassay using anti-DIG-AP and the chemiluminescent substrate CSPD. The chemiluminescent signals were captured with Molecular Imager Device (Bio-Rad). The sequences of the probes are the following: IFI16 wild-type, 5'-AGAATAGGAGCAAGCCAGCACTAGTCAGCTAAC-3' (forward) and 5'-GTTAGCCTACGTGCTTGGCTGCC- TATTCT-3' (backward); IFI16 mutant, 5'-AGAATAGGAGTACCCAGCATTACTCGAATA-3' (forward) and 5'-GTTAGCCTACGTGCTTGGCTGCC- TATTCT-3' (backward); and p53 DNA-binding consensus sequence, 5'-TATTACAGAACATGTCAAGCTATGGGG-3' (forward) and 5'-CCCAAGCATGTTAGACATGTCTAATA-3' (backward).

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

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