p53 Deficiency Leads to Compensatory Up-Regulation of p16INK4a

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Abstract
p53-p21-cyclin-dependent kinase and p16INK4a-cyclin-dependent kinase pathways have parallel functions in preventing tumorigenesis. In cancer patients, tumor suppressor p53 is frequently inactivated through mutations, whereas p16INK4a is silenced through promoter methylation. However, the interaction between these two pathways is less well understood. Here, we report that p53 controls p16INK4a expression in a unique way. p53 deficiency led to up-regulation of p16INK4a in primary mouse embryonic fibroblasts, osteoblasts, and various mouse organs, and an increase in the p16INK4a promoter activity, without affecting the half-life of p16INK4a. Reconstitution of p53, but not mutant p53, restored the proper expression of p16INK4a. These results indicate that p53 is necessary in repressing p16INK4a expression. However, up-regulation of p53 in response to genotoxic stress or nutlin-3 treatment did not down-regulate p16INK4a. p53 did not repress the p16INK4a promoter activity either. These findings suggest that p53 has a necessary but not sufficient role in repressing p16INK4a expression. p16INK4a elevation in p53-/- cells is, at least partially, mediated by Ets1, a known positive regulator of p16INK4a, as p53 deficiency up-regulated Ets1 through protein stabilization and knockdown of Ets1 down-regulated p16INK4a expression in p53-/- mouse embryonic fibroblasts. These studies uncover a compensatory mechanism for the loss of p53 and provide a basis for targeting both p53 and p16INK4a in cancer therapy. (Mol Cancer Res 2009;7(3):354–60)

Introduction
p53 is a tumor suppressor that is mutated in >50% of the human primary cancers (1, 2). It acts as a transcription activator or repressor to control the transcription of target genes involved in cell proliferation, apoptosis, or differentiation (2, 3). Activation of p53 can be induced by genotoxic and other types of stress. The best studied examples of p53 target genes include p21, GADD45, Bax, and Puma. p21 mainly binds to cyclin-dependent kinase (CDK) 2-cyclin A/E and possibly CDK4/6-cyclin D complexes (2). Another CDK inhibitor, p16INK4a, which has no sequence homology to p21, mainly binds to CDK4 or CDK6 and forms a binary complex. Binding of p21 to CDK-cyclin complexes or binding of p16INK4a to CDKs inhibit the CDK activities and lead to cell cycle exit (4). It has been found that p16INK4a is also frequently lost in many types of cancers through promoter hypermethylation (5).

Mouse genetic studies revealed that p53-/- mice develop mostly lymphomas and osteosarcomas at 3 to 6 months, whereas p16INK4a-/- mice develop lymphoma, sarcomas, and melanomas (1, 2, 6). However, mice deficient for both p16INK4a and p53 show an increase in tumorigenesis rate and in an expansion of the tumor spectrum. It is thus proposed that p53 and p16INK4a function in distinct anticancer pathways but might have some redundant functions (7, 8). At the cell level, p53 and p16INK4a are reported to act in parallel to promote senescence of primary cells (9). In some cancer types such as head and neck cancer, a concurrent “loss of function” mutation of p53 and loss of expression of p16INK4a has been observed (10). However, in other cancer types such as biliary tract cancer, no such link has been observed (11).

Here, we studied the possible link between the p53-p21 pathway and the p16INK4a-CDK pathway by analyzing primary cells that were freshly isolated from p53-/- or p16INK4a-/- mice in comparison with normal cells isolated from their control litters. This is to avoid the use of immortalized or transformed cells as they usually contain mutations in these two pathways. Our studies identify a unique mechanism by which p53 regulates p16INK4a expression, thus establishing a crosstalk between the two important tumor suppression pathways.

Results
Up-Regulation of p16INK4a at the Transcription Level in p53-/- Primary Cells
To study possible interaction between the p53-p21 and the p16INK4a-Rb pathways, we analyzed the protein levels of p16INK4a in p53-/- mouse embryonic fibroblasts (MEF) in comparison with normal MEFs that were freshly isolated from day 13.5 embryos of the same litter. This cannot be done with tumor cell lines as they frequently lose p16INK4a expression due to promoter methylation. Because p16INK4a expression is affected by growth conditions, we cultured wild-type and p53-/- MEFs for 1, 2, or 4 days. Western blot analysis revealed a

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marked increase in the protein levels of p16\textsuperscript{INK4a} in p53\textsuperscript{-/-} MEFs under these growth conditions (Fig. 1A and B). Similar results were obtained from primary osteoblasts that were isolated from the calvarias of day 1 pups (Fig. 1C). These results indicate that p53 deficiency led to an up-regulation of p16\textsuperscript{INK4a} and suggest that p53 might directly or indirectly repress p16\textsuperscript{INK4a} expression.

We next reconstituted p53\textsuperscript{-/-} MEFs with wild-type or mutant p53 that lack DNA-binding activity. The cells were infected with retroviruses that express these proteins and then selected against

![FIGURE 1](image_url)

**FIGURE 1.** p53 deficiency led to up-regulation of p16\textsuperscript{INK4a} in primary MEFs and osteoblasts. **A.** Western blot shows elevated p16\textsuperscript{INK4a} protein levels in p53\textsuperscript{-/-} MEFs compared with control cells that were cultured for 1 to 4 d. **B.** Quantitation data of **A.** **C.** Western blot shows elevated p16\textsuperscript{INK4a} protein levels in p53\textsuperscript{-/-} osteoblasts compared with control cells that were cultured for 1 to 4 d. **D.** Protein levels of p16\textsuperscript{INK4a} were reduced in p53\textsuperscript{-/-} MEFs that expressed wild-type p53 but not p53Arg273His. Note that wild-type p53 could not be expressed to high levels.

![FIGURE 2](image_url)

**FIGURE 2.** Up-regulation of p16\textsuperscript{INK4a} occurred at the level of transcription. **A.** Half-life of p16\textsuperscript{INK4a} was not altered by p53 deficiency. Wild-type and p53\textsuperscript{-/-} MEFs were treated with 10 \( \mu \)g/mL cycloheximide for different periods and then harvested. Protein levels of p16\textsuperscript{INK4a} were determined by Western blot. **B.** Quantitation data for **A** to show that p16\textsuperscript{INK4a} had a normal half-life in p53\textsuperscript{-/-} MEFs. Basal levels of p16\textsuperscript{INK4a} at time 0 for wild-type and p53\textsuperscript{-/-} cells were set at 1.0. **C.** RT-PCR shows elevated p16\textsuperscript{INK4a} at the mRNA levels in p53\textsuperscript{-/-} MEFs. **D.** Reporter assay shows that the p16\textsuperscript{INK4a} promoter activity was enhanced in p53\textsuperscript{-/-} MEFs. p16\textsuperscript{INK4a}-luciferase construct was transfected into wild-type or p53\textsuperscript{-/-} MEFs and luciferase activities were determined.
puromycin for 3 to 4 days. The protein levels of p16\textsuperscript{INK4a} and p53 were analyzed with Western blot. As expected, expression of wild-type p53 brought down the levels of p16\textsuperscript{INK4a}, whereas the mutant p53 failed to do so (Fig. 1D). Note that the level of wild-type p53 is extremely low. This is likely due to the cytotoxic effect of p53 so that only cells with low expression of p53 could survive. These results suggest that loss of functional p53 that occurs in human cancer patients, such as p53 deficiency, might turn on p16\textsuperscript{INK4a} expression and that this up-regulation requires p53 to bind to its cis-element to regulate transcription of p16\textsuperscript{INK4a} or a protein that mediates p16\textsuperscript{INK4a} up-regulation.

To understand the nature of this up-regulation, we measured the half-lives of p16\textsuperscript{INK4a} after stopping translation with cycloheximide treatment. No significant difference was observed between wild-type and p53\textsuperscript{−/−} MEFs (Fig. 2A and B), suggesting that p53 did not affect the stability of p16\textsuperscript{INK4a}. We then measured the p16\textsuperscript{INK4a} mRNA levels with real-time PCR (RT-PCR) and found that p53 deficiency led to an elevation of p16\textsuperscript{INK4a} at the mRNA level (Fig. 2C). Moreover, p16\textsuperscript{INK4a} promoter, when transfected into p53\textsuperscript{−/−} MEFs, showed higher activities than in wild-type cells (Fig. 2D). These results indicate that p53 directly or indirectly represses p16\textsuperscript{INK4a} expression.

Cancer development is frequently accompanied by the loss of p53 function in various tissues. p53 dysfunction is believed to be a major contributor to cell transformation in many cell types as well. To test whether p16\textsuperscript{INK4a} expression is regulated by p53 deficiency \textit{in vivo}, we analyzed the mRNA levels of p16\textsuperscript{INK4a} in several tissues of p53\textsuperscript{−/−} mice in comparison with their littermate controls. RT-PCR analysis confirmed that p16\textsuperscript{INK4a} was up-regulated in most of the p53\textsuperscript{−/−} mouse organs tested (Fig. 3), suggesting that cells would up-regulate the transcription of p16\textsuperscript{INK4a} when p53 is lost or mutated \textit{in vivo}.

\textbf{A Necessary but not Sufficient Role for p53 in Repressing p16\textsuperscript{INK4a} Expression}

The above results indicate that p53 directly or indirectly represses the transcription of p16\textsuperscript{INK4a}. To distinguish these two possibilities, we tried to elevate the p53 protein levels and then analyze the effect on p16\textsuperscript{INK4a} expression. We first tried to express p53 in primary wild-type MEFs using retrovirus and found that, after 2 to 3 days of selection against puromycin, the levels of p53 protein was very low, as we have observed in

\begin{figure}[h]
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\includegraphics[width=\textwidth]{fig3}
\caption{RT-PCR shows an elevation of p16\textsuperscript{INK4a} mRNA levels in various organs of p53\textsuperscript{−/−} mice compared with wild-type control mice. Organs were isolated from adult p53\textsuperscript{−/−} and wild-type mice of the same litter. Total RNA was isolated and then used to determine the mRNA levels of p16\textsuperscript{INK4a} with RT-PCR.}
\end{figure}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig4}
\caption{p53 was not sufficient in repressing p16\textsuperscript{INK4a} expression. A. DNA damage reagent Adriamycin up-regulated the protein levels of p53 but failed to markedly repress p16\textsuperscript{INK4a}. B. An increase in p53 induced by nutlin-3 did not significantly repress p16\textsuperscript{INK4a} in MEFs. C. Reporter assay showing the effect of p53, mutant p53, or Ets1 on the promoter activity of p16\textsuperscript{INK4a} in coexpression experiment.}
\end{figure}
p53<sup>−/−</sup> MEFs (Fig. 1D). No significant change in the levels of p16<sup>INK4a</sup> was observed either (data not shown). We then used the genotoxic drug Adriamycin to up-regulate p53. The increase in the p53 protein level was enormous in the presence of Adriamycin; however, the protein levels of p16<sup>INK4a</sup> were not significantly reduced in MEFs (Fig. 4A). Moreover, p16<sup>INK4a</sup> levels were not reduced by treatment with nutlin-3, which specifically disrupts the interaction between Mdm2 and p53 and leads to an elevation of p53 without causing DNA damage (Fig. 4B). The p16<sup>INK4a</sup> promoter has no consensus p53 binding sites and coexpression of p53 did not significantly affect the p16<sup>INK4a</sup> promoter activity in a luciferase reporter assay (Fig. 4C). These results suggest that p53 did not directly regulate p16<sup>INK4a</sup> transcription and that p53 is necessary but not sufficient in repressing p16<sup>INK4a</sup> expression, at least in a short-term. This mode of regulation ensures that, in response to DNA damage, p16<sup>INK4a</sup> expression is maintained. Only when p53 is dysfunctional, p16<sup>INK4a</sup> expression is activated.

As shown previously, we found that Ets1 was able to activate the p16<sup>INK4a</sup> promoter in luciferase reporter assays, although to a modest level (data not shown). To show that elevated Ets1 was responsible for up-regulation of p16<sup>INK4a</sup> in p53<sup>−/−</sup> cells, we knocked down Ets1 with pooled small interfering RNA, which resulted in a 5.8-fold reduction at the mRNA level but only a 2.5-fold reduction at the protein level (Fig. 5D). We also observed a 2.2-fold decrease in the protein levels of p16<sup>INK4a</sup> (Fig. 5D). However, knockdown of Ets2 showed a much less effect on the protein level of p16<sup>INK4a</sup> and knockdown of both Ets1 and Ets2 showed a similar effect as knockdown of Ets1 alone (Fig. 5D). This less than complete reduction in the p16<sup>INK4a</sup> levels could be due to the redundant functions provided by other members of the ETS family or transcription factors that are not related to ETS. These results are consistent with the finding that cells deficient for Ets1 showed marked decrease in p16<sup>INK4a</sup> and indicate that up-regulated expression of p16<sup>INK4a</sup> in p53<sup>−/−</sup> cells was, at least partially, mediated by up-regulation of Ets1.

**Ets1 Was Stabilized in p53<sup>−/−</sup> Cells**

How does p53 deficiency up-regulate the protein levels of Ets1? RT-PCR analysis indicated that Ets1 mRNA level was not altered in p53<sup>−/−</sup> MEFs (Fig. 6A). RT-PCR analysis revealed no alteration in the levels of Ets1 mRNA in p53<sup>−/−</sup> mouse organs (Fig. 6B). Moreover, reporter assay confirmed that p53 showed no effect on the promoter activity of Ets1 (data not shown). This is in disagreement with a previous report showing that p53 could repress Ets1/2 promoter activity and that p53 was present in the transcription complex assembled on the Ets1 promoters (16). Instead, we found that Ets1 was stabilized in p53<sup>−/−</sup> MEFs (Fig. 6C). It has been reported that p53 could regulate the
stability of several proteins such as PTEN and Cdc6, although the mechanisms are not well understood (17, 18). Taken together, these results indicate that p53 regulates Ets1 mainly at the post-translational level at least in MEFs. Ets1 protein stabilization in the absence of p53 might explain why small interfering RNA was not very efficient (Fig. 5D). Further investigation will be needed to determine the molecular mechanisms by which p53 regulates the protein stability of Ets1.

**p16INK4a Deficiency Had a Modest Effect on p53 Expression in MEFs**

Having shown a role for p53 in repression of p16INK4a expression, we then asked whether the reciprocal relationship exists in MEFs. We used p16INK4a-/- MEFs and cultured them for different periods and tested the protein levels of p53. A modest up-regulation of p53 was observed in p16INK4a-/- MEFs (Fig. 7A and B). An insignificant change in the mRNA levels of p53 was also observed. These results suggest that p16INK4a deficiency has a modest effect on p53 expression.

**Discussion**

p53 plays a crucial role in controlling cell proliferation and death. Loss of p53 function has been found in >50% of the human primary cancers. In addition, p53 has been reported to play important roles in cell differentiation. Loss of p53 affects bone, kidney, and brain development (2, 3). It is conceivable that cells must have evolved certain mechanisms to compensate for the loss of p53. Using mouse tissues and cells, we, for the first time, identified p16INK4a, another important tumor suppressor, as one of such pathways. In cells that have lost p53, this up-regulation presumably helps to curb cell over-proliferation. This might put the selection pressure on p16INK4a, as silencing p16INK4a or acquisition of mutations in the p16INK4a pathway will confer the cells with more growth advantage. This provides a possible explanation for the observation that some of the cancers show concurrent mutations in p53 and the silencing of p16INK4a and that mice deficient for both p53 and p16INK4a show accelerated tumor formation (10, 19). This finding also provides a theoretical basis for targeting both p53 and p16INK4a in cancer therapy.
Whereas p53 deficiency leads to p16INK4a up-regulation, we found that p16INK4a deficiency has a very modest effect on p53 expression in MEFs. However, during our studies, it was reported that p16INK4a inversely modulates the p53 protein levels through p14ARF/Mdm2-mediated degradation of p53 in human mammary epithelia cells but not in primary human mammary fibroblasts (20). When p16INK4a is knocked down with shRNA in SiHa and Ca Ski cells, p53 is up-regulated (21). However, we found that p53 deficiency-induced p16INK4a elevation is much higher than p16INK4a deficiency-induced p53 elevation in primary cells. These results, taken together, suggest that there exists an interplay between the p53-p21 and the p16INK4a-Rb pathways in constraining cell proliferation.

How does p53 deficiency up-regulate p16INK4a expression? As discussed previously, p16INK4a expression can be regulated by transcription regulators including Ets1/2 and Id1. In addition, p16INK4a promoter activity can be silenced by hypermethylation. One possibility is that p53 deficiency might alter the methylation of p16INK4a promoter and this will need further investigation. Another possibility is that p53 deficiency affects the expression of Ets1/2 and/or Id1. Indeed, our studies here indicate that Ets1 is the transcription factor that can link p53, instead of regulating the transcription of Ets1, controls the protein stability of Ets1. Stability of Ets1 could be controlled by protein(s) of the ubiquitin-proteasome pathway whose expression is regulated by p53.

Materials and Methods

Isolation and Culture of Primary MEFs and Calvarial Osteoblasts

Primary MEFs were isolated from 13.5-day-old embryos and osteoblasts were isolated from the calvaria of day 1 pups following previously reported protocols (2, 3). MEFs were cultured in DMEM and osteoblasts were cultured in α-MEM supplemented with 10% fetal bovine serum. The mice were kept at the Biological Resources Center of Singapore and used for the experiments following the protocols approved by the Institute of Molecular and Cell Biology and Biological Resources Center.

Western Blot Analysis

The cells were rinsed twice with ice-cold PBS and harvested by scraping in radioimmunoprecipitation assay buffer. The proteins were quantified using DC protein assay (Bio-Rad). Proteins (60 μg) were resolved by SDS-PAGE and the levels of proteins were determined by Western blot analysis using β-actin as a control. The primary antibodies used for analysis were anti-p53 and phospho-p53 (Cell Signaling), anti-Ets1, Ets2, Id1, and p16INK4a (Santa Cruz Biotechnology), and anti-β-actin (Sigma-Aldrich).

RNA Isolation and Quantitative RT-PCR

Total RNA was extracted from cells or mouse organs with TRIzol reagent (Invitrogen) according to the manufacturer's protocol. cDNAs were synthesized from 1 μg total RNA using Reverse-It RTase Blend (Abgene) with random hexamers. The cDNA was then analyzed by quantitative RT-PCR using the LightCycler 480 Probes Master Kit (Roche) on a LightCycler 480 RT-PCR System (Roche). The primer sequences and specific universal probes used for quantitative RT-PCR are available on Roche Applied Science Web site.

Promoter Studies and Luciferase Assay

MEFs or 293 cells were seeded on 12-well plates. pGL2- and pGL3-basic vector or and constructs containing full-length Ets1 promoter and p16INK4a promoter, as well as p53 or Ets1, were cotransfected with pRL-null into the cells and harvested after 48 h. Cells were lysed with passive lysis buffer from Promega and subjected to a single freeze-thaw cycle. The lysates were assayed for both firefly and Renilla luciferase (as normalization control) activities using Dual-Luciferase Reporter Assay System (Promega) on a GloMax 96 Microplate Luminometer (Promega).

Retrovirus Infection and Knockdown Experiment

Recombinant retroviruses were produced by using the Plat E packaging cell line that was transfected with the pMSCV vector as well as constructs expressing p53 or p53Arg273His. The viral supernatant was collected 48 h later and used to infect p53-/- MEFs. The infected cells were subjected to puromycin selection at a concentration of 2 μg/mL at day 2 after infection before harvesting at day 5 or 6. To knock down Ets1 or Ets2, we used ON-TARGETplus SMARTpool small interfering RNA and reagents from Dharacon following the manufacturer’s protocol. The small interfering RNA sequences are available on Dharacon Web site.

Quantitation and Statistical Analysis

Western blots were scanned with a Molecular Dynamics scanning densitometer. The relative levels of proteins of interest were determined by measuring the intensity of the corresponding bands. All values were averages of three experiments and were normalized to the level of β-actin. Statistical analysis was done using an unpaired t test (Statistica software; StatSoft). Significant association was defined when P < 0.05 compared with control.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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