

# The Potential Tumor Suppressor p73 Differentially Regulates Cellular p53 Target Genes<sup>1</sup>

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## Abstract

p73, a potential tumor suppressor, is a p53 homologue. Transient over expression of p73 in cells can induce apoptosis and p21, a cellular p53 target gene primarily responsible for p53-dependent cell cycle arrest. To further characterize the role of p73 in tumor suppression, we established several groups of cell lines that inducibly express p73 under a tetracycline-regulated promoter. By using these cell lines, we found that p73 can induce both cell cycle arrest and apoptosis. We also found that p73 can activate some but not all of the previously identified p53 cellular target genes. Furthermore, we found that the transcriptional activities of p53, p73 $\alpha$ , and p73 $\beta$  to induce their common cellular target genes differ among one another. These results suggest that p73 is both similar to and different from p53 in their signaling pathways leading to tumor suppression.

## Introduction

Cytogenetic and molecular genetic analyses have shown that loss of heterozygosity in chromosome 1p36 was frequently found in neuroblastoma (1). Recently, p73, a homologue of the well-defined tumor suppressor p53 (2), was found to lie within the chromosome 1p36 region, suggesting that it may be a new tumor suppressor (2). The p73 gene is expressed as p73 $\alpha$ , a 636-amino acid polypeptide, and p73 $\beta$ , a 499-amino acid polypeptide that is encoded by an alternatively spliced transcript lacking the 96 nucleotides corresponding to exon 13. The homology between p53 and p73 is extensive within the most conserved p53 domains (*i.e.*, activation, sequence-specific DNA binding, and oligomerization).

As a p53 homologue, it is reasonable to suggest that p73 may have biological activities similar to that of p53. Indeed, p73 was shown to be capable of activating the p21 cyclin-dependent kinase inhibitor gene (2, 3), a well-defined cellular p53 target primarily responsible for p53-dependent G<sub>1</sub> arrest (4). However, it is not clear whether p73 can induce other cellular p53 targets that also cause growth suppression or cell cycle arrest, *e.g.*, 14-3-3 $\sigma$  (5) and BTG2 (6). Apoptotic activity is the other function that is conserved between p53 and p73, although it is not clear how p73 induces apoptosis. Although several hypotheses for the mechanism of p53-dependent apoptosis have been proposed, it is still not certain how p53 induces apoptosis (7). Many studies show that p53 transcriptional activity contributes to its ability to induce apoptosis (7). There are several candidate genes that play roles in apoptosis that can be activated by p53 *e.g.*, BAX (8), KILLER/DR5 (9), several redox-related genes (PIGs; Ref. 10), and the p85 regulatory subunit of the signaling protein phosphatidylyl-3-OH kinase (11). In this study, we provide evidence that despite the high similarity in the

activation and DNA binding domains between p53 and p73, the p53 and p73 signaling pathways leading to tumor suppression are both similar and different.

## Materials and Methods

**Plasmids.** cDNAs for p73 $\alpha$ , p73 $\beta$ , and p73 $\alpha$ 292 (Ref. 3; kindly provided by W. Kaelin) were cloned separately into a tetracycline-regulated expression vector, 10-3, at its Eco RI and Xba I sites, and the resulting plasmids were used to generate cell lines that inducibly express p73. p73 proteins were tagged at their N-termini with influenza hemagglutinin peptide.

**Generation of H1299 Cell Lines that Inducibly Express p73.** H1299 cell lines that inducibly express p73 were generated as described previously (12). Individual clones were screened for inducible expression of p73 protein by Western blot analysis using monoclonal antibody 12CA5. The H1299 cell line that inducibly expresses wild-type p53 is p53-3 as described previously (12).

**Western Blot Analysis.** Cells were collected from plates in PBS, resuspended with 1  $\times$  sample buffer, and boiled for 5 min. Western blot analysis was performed as described previously (12). Affinity purified anti-actin polyclonal antibodies was purchased from Sigma Chemical Co. (St. Louis, MO). 12CA5 was purchased from Boehringer Mannheim Biochemicals (Indianapolis, IN).

**Growth Rate Analysis.** To determine the rate of cell growth, cells were seeded at approximately  $7.5 \times 10^4$  cells/60-mm plate, with or without tetracycline (1  $\mu$ g/ml). The medium was replaced every 72 h. At times indicated, three plates were rinsed with PBS twice to remove dead cells and debris. Live cells on the plates were trypsinized and collected separately. Cells from each plate were counted three times using the Coulter cell counter. The average number of cells from three plates were used for growth rate determination.

**FACS Analysis.** Cells were seeded at  $2.0 \times 10^5$ /90-mm plate, with or without tetracycline. Three days after plating, both floating dead cells in the medium and live cells on the plate were collected and fixed with 2 ml of 70% ethanol for at least 30 min. The fixed cells were centrifuged and resuspended in 1 ml of PBS solution containing 50  $\mu$ g/ml each of RNase A (Sigma Chemical Co.) and propidium iodide (Sigma Chemical Co.). The stained cells were analyzed in a FACS<sup>4</sup> sorter (FACSCaliber; Becton Dickinson) within 4 h. The percentage of apoptotic cells containing a sub-G<sub>1</sub> DNA content was quantitated using the CellQuest program. The percentage of live cells in G<sub>0</sub>-G<sub>1</sub>, S, and G<sub>2</sub>-M phases was quantitated using the ModFit program.

**RNA Isolation and Northern Blot Analysis.** Total RNA was isolated using Trizol reagents (Life Technologies, Inc.). Northern blot analysis was performed as described (13). The p21, MDM2, BAX, GADD45, and glyceraldehyde-3-phosphate dehydrogenase probes were prepared as described previously (13). The MCG-B61, MCG-B69, and MCG-B71 cDNA probes were PCR fragments identified by CLONTECH PCR-Select cDNA Subtraction assay.<sup>5</sup> The KILLER/DR5 cDNA probe (GenBank #159553) was purchased from American Type Culture Collection. The following cDNA probes were purchased from Genome System, Inc. (St. Louis, MO): BTG2 (GenBank #H86711), 14-3-3 $\sigma$  (W79136), PIG1 (W61024), PIG2 (H18355), PIG3 (N75824), PIG4 (H45773), PIG6 (R88591), PIG8 (R42786), PIG10 (R87338), PIG11 (R54648), PIG12 (AA149234), and p85 (N21330).

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<sup>4</sup> The abbreviation used is: FACS, fluorescence-activated cell sorting.

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## Results

**p73 Can Induce Cell Cycle Arrest and Apoptosis.** Previously, we have established a number of H1299 non-small cell lung carcinoma cell lines that inducibly express p53 under a tetracycline-regulated promoter and showed that p53 can induce either cell cycle arrest or apoptosis (12). By using a similar approach, we generated several H1299 cell lines that inducibly express either p73 $\alpha$ , p73 $\beta$ , or p73 $\alpha$ 292. p73 $\alpha$ 292(R292H) was previously shown to be defective in inducing growth suppression and apoptosis (2, 3). Two representative cell lines each that express either p73 $\alpha$ , p73 $\beta$ , or p73 $\alpha$ 292 are shown in Fig. 1. p73 $\alpha$ -22 expresses a high level of p73 $\alpha$  when induced (+), whereas p73 $\alpha$ -4 produces a moderate level of p73 $\alpha$  (Fig. 1A). p73 $\beta$ -9 expresses a high level of p73 $\beta$  when induced (+), whereas p73 $\beta$ -26 produces a moderate level of p73 $\beta$  (Fig. 1B). p73 $\alpha$ 292-20 expresses a high level of p73 $\alpha$ 292 when induced (+), whereas p73 $\alpha$ 292-3 produces a low level of p73 $\alpha$ 292 (Fig. 1C). The amount of actin was also quantitated as an internal control (Fig. 1, A–C, bottom).

To characterize the activity of p73, growth rates of p73 $\alpha$ -22, p73 $\beta$ -9, and p73 $\alpha$ 292-20 cell lines were determined. Dramatic differences were seen for wild-type p73, but not mutant p73 $\alpha$ 292-expressing cells, when the growth rates of uninduced and induced cells were compared (Fig. 2, A, D, G, and J). As shown in Fig. 2, A and D, cells that did not express p73 continued to grow (○, uninduced), whereas both the p73 $\alpha$ - and p73 $\beta$ -expressing cells failed to multiply and started to die within 2 days on induction (▲, induced). These results are consistent with previous reports that p73 $\alpha$  and p73 $\beta$  can cause growth suppression (2, 3). It should be mentioned that we also tested two other wild-type p73-expressing cell lines, p73 $\alpha$ -4 and p73 $\beta$ -26. Results from these two cell lines were similar to that from p73 $\alpha$ -22 and p73 $\beta$ -9 (data not shown). In contrast, the growth rates of p73 $\alpha$ 292-20 cells that inducibly express p73 $\alpha$ 292 were nearly identical under both the uninduced (□) and induced (◇) conditions (Fig. 2G). As control, the growth rates of H1299-24 cells that were similarly established but did not express any protein were nearly identical in the presence (□ +tet) or absence (◇ –tet) of tetracycline (Fig. 2J).

Next, we used FACS analysis to examine whether p73 can induce apoptosis and/or cell cycle arrest. Previously, we have shown that the percentage of cells containing a sub-G<sub>1</sub> content of DNA reflects the extent to which cells are undergoing apoptosis (12, 13). When p73 was not expressed, the percentage of p73 $\alpha$ -22 and p73 $\beta$ -9 cells that had a sub-G<sub>1</sub> content of DNA were 2% and 1%, respectively (Fig. 2, B and E). However, when p73 $\alpha$  or p73 $\beta$  was induced, the percentage of p73 $\alpha$ -22 and p73 $\beta$ -9 cells that had a sub-G<sub>1</sub> content of DNA was increased to 20% and 44%, respectively (Fig. 2, C and F). These results indicate that the p73-expressing cells were undergoing apoptosis and confirm the previous report that p73 can induce apoptosis in the SAOS-2 osteosarcoma cell line (3). Furthermore, FACS analysis showed that the number of live cells containing S phase amounts of

DNA was substantially decreased when p73 $\alpha$  or p73 $\beta$  was expressed (compare Fig. 2, B with C, and Fig. 2, E with F). Quantitation by the Modfit program showed that the S phase cells were decreased from 35–12% for the p73 $\alpha$ -22 cell line and from 34–20% for the p73 $\beta$ -9 cell line. In addition, the G<sub>0</sub>-G<sub>1</sub> and G<sub>2</sub>-M phase cells were increased on p73 expression. The G<sub>0</sub>-G<sub>1</sub> phase cells were increased from 51–62% for the p73 $\alpha$ -22 cell line (compare Fig. 2, B with C) and from 52–58% for the p73 $\beta$ -9 cell line (compare Fig. 2, E with F). The G<sub>2</sub>-M phase cells were increased from 15–26% for the p73 $\alpha$ -22 cell line (compare Fig. 2, B with C) and from 14–22% for the p73 $\beta$ -9 cell line (compare Fig. 2, E with F). These results indicated that the p73-expressing cells also underwent cell cycle arrest and the arrest occurred in both G<sub>1</sub> and G<sub>2</sub>-M. It should be mentioned that we also tested p73 $\alpha$ -4 and p73 $\beta$ -26 cell lines. Results from these two cell lines were similar to that from p73 $\alpha$ -22 and p73 $\beta$ -9 (data not shown). In contrast, induction of mutant p73 $\alpha$ 292 or withdrawal of tetracycline had no effect on the cell cycle distribution of p73 $\alpha$ 292-20 cells (compare Fig. 2, H with I) and H1299-24 cells (compare Fig. 2, K with L), respectively.

**Common and Distinctive Cellular Target Genes Activated by p53 and p73.** Because the DNA binding domain in p73 is 63% identical to that in p53, it is reasonable to suggest that p73 can activate p53 target genes. We found that some p53 cellular target genes can be activated, but not others, by p73 (Fig. 3). In addition, most of the genes that can be activated by p73 were only weakly induced by p73 (Table 1). Interestingly, *14-3-3 $\sigma$*  and *PIG7* were induced by p73 to an extent that is substantially greater than by p53 (Table 1).

Previous reports have shown that the p21 promoter can be activated by p73 and that the level of p21 protein was increased in cells when exogenous p73 was expressed (2, 3), suggesting that the *p21* gene may also be a cellular target of p73. Consistent with these results, Northern blot analysis showed that p21 was induced by p73 $\alpha$  and p73 $\beta$  in p73 $\alpha$ -22 and p73 $\beta$ -9 cells, but not by mutant p73 $\alpha$ 292 in p73 $\alpha$ 292-20 cells (Fig. 3A). As expected, p21 was also induced by p53 in p53-3 cells when p53 was expressed (Fig. 3A). However, PhosphorImage quantitation showed that the level of p21 induced by p53 is at least three and six times higher than by p73 $\beta$  and p73 $\alpha$ , respectively (Table 1). The results suggest that p53, p73 $\alpha$ , and p73 $\beta$  may differ from each other in transcriptional activity, which is further supported by additional Northern blot analyses (see below).

Next, we determined whether p73 can regulate *MDM2*, *GADD45*, *BAX*, *BTG2*, *14-3-3 $\sigma$* , *p85*, and *KILLER/DR5*. We found that *MDM2*, an oncogene and a negative regulator of p53 (14), was significantly induced by p73 $\beta$ , albeit much less than by p53 (Fig. 3B). However, *MDM2* was induced little, if any, by p73 $\alpha$ . *GADD45*, a DNA damage responsive gene involved in DNA repair (15), was slightly activated by p73 (Fig. 3C). *BAX*, an apoptosis activator that can be activated by p53 (8), was induced little, if any, by p73 (Fig. 3D). *BTG2*, a nerve growth factor responsive gene that can cause growth suppression (6), was weakly activated by p73 (Fig. 3E). Surprisingly, *14-3-3 $\sigma$* , a gene that might mediate p53-dependent G<sub>2</sub>-M arrest, was induced by p73 $\alpha$  and p73 $\beta$  to an extent that is six and two times greater than by p53, respectively (Fig. 3F). *p85* is a regulatory subunit of the signaling protein phosphatidylinositol-3-OH kinase that might be involved in the p53-dependent apoptotic response to oxidative stress (11). On H<sub>2</sub>O<sub>2</sub> treatment, the level of p85 was increased in a p53-dependent manner (11). We found that p85 was induced 2–3-fold by either p53, p73 $\alpha$ , or p73 $\beta$ , but not p73 $\alpha$ 292 (Fig. 3G; Table 1). *KILLER/DR5*, a death receptor gene that can be induced by genotoxic stress and p53 (9), was weakly induced by p53 and p73 $\beta$  but little, if any, by p73 $\alpha$  (Fig. 3H; Table 1).



Fig. 1. Characterization of cell lines that inducibly express p73. A, levels of p73 $\alpha$  (top) and actin (bottom) in p73 $\alpha$ -4 and p73 $\alpha$ -22 cell lines were assayed by Western blot analysis. Cell extracts were prepared from uninduced cells (–) and cells induced to express (+) p73 $\alpha$ . The upper portion of the blot was probed with anti-hemagglutinin antibody 12CA5, and the bottom portion of the blot was probed with anti-actin polyclonal antibody. B, levels of p73 $\beta$  and actin in p73 $\beta$ -9 and p73 $\beta$ -26 cell lines. The experiments were performed similar to that in A. C, levels of p73 $\alpha$ 292 and actin in p73 $\alpha$ 292-3 and p73 $\alpha$ 292-20 cell lines. The experiments were performed similar to that in A.

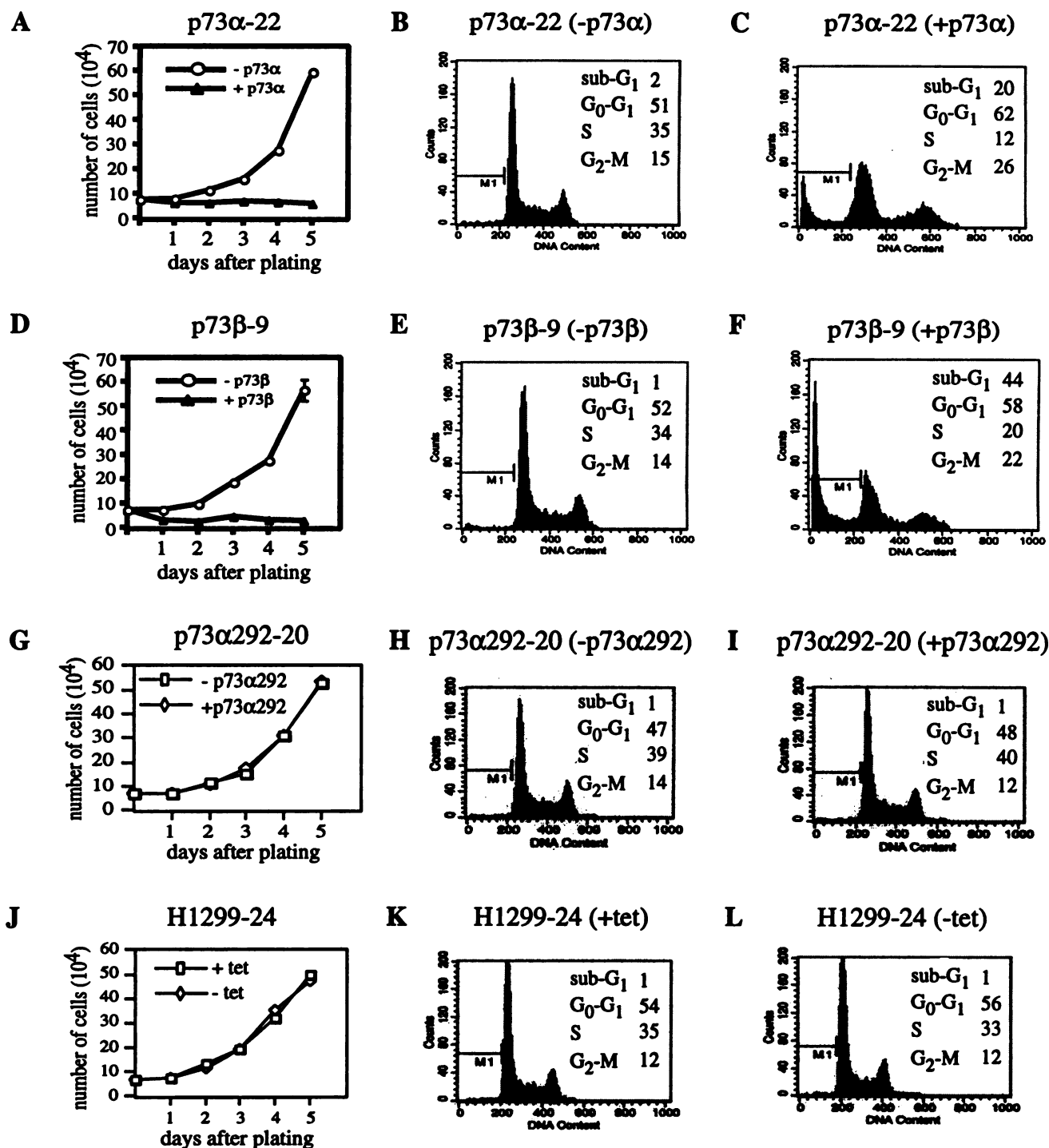


Fig. 2. p73 can induce both cell cycle arrest and apoptosis. A, D, G, and J, growth rates of p73-expressing and control cells under both the uninduced and induced conditions were measured as described in "Materials and Methods." B, C, E, F, H, I, K, and L, DNA contents of p73-expressing and control cells on day 3 under both the uninduced (B, E, H, and K) and induced (C, F, I, and L) conditions were quantitated by propidium iodide staining and FACS analysis as described in "Materials and Methods." M<sub>1</sub> represents cells that contain a sub-G<sub>1</sub> content of DNA. The percentage of apoptotic cells containing a sub-G<sub>1</sub> content of DNA under both the uninduced and induced conditions was quantitated using CellQuest program. The percentage of live cells in G<sub>0</sub>-G<sub>1</sub>, S, and G<sub>2</sub>-M under both the uninduced and induced conditions was quantitated using ModFit program.

Several redox-related genes (PIGs), that were shown to be activated by p53 (10), were examined for p73 induction. We confirmed that *PIG2*, *PIG3*, *PIG6*, *PIG7*, *PIG8*, and *PIG11* were significantly induced by p53 in H1299 cells (Fig. 3, I-N). However, we found that *PIG3*, *PIG6*, and *PIG11* were not significantly induced by p73 (Fig. 3, J, K, and N) and *PIG2* was slightly induced by p73 $\alpha$  but not by p73 $\beta$  (Fig. 3I). *PIG8* was induced by both p73 $\alpha$  and p73 $\beta$  to a level

slightly less than by p53 (Fig. 3M). *PIG7* was substantially induced by p73 $\beta$  to a level that is two times more than by p53 (Fig. 3L). We also found that *PIG10* and *PIG12* were not substantially induced by p53, and *PIG1* and *PIG4* were undetectable in H1299 cells (data not shown). Therefore, p73 induction of the *PIG1*, *PIG4*, *PIG10*, and *PIG12* genes was not analyzed.

We also examined three potential p53 target genes we recently

identified, *MCG-B61*, *MCG-B69*, and *MCG-B71*,<sup>5</sup> for p73 induction. *MCG-B61* was induced by p73 $\beta$ , but not by p73 $\alpha$  (Fig. 3O). *MCG-B69* was significantly induced by p73 (Fig. 3P). However, *MCG-B71* was not induced by p73 (Fig. 3Q).

## Discussion

The cell lines described above provide several novel observations. Similar to p53, p73 can induce cell cycle arrest and apoptosis in H1299 cells. We also demonstrate that although p73 is homologous to p53 in the activation and DNA binding domains, p73 differentially activate some but not all p53 cellular target genes.

Our data provide evidence that p73 can induce cell cycle arrest. FACS analysis showed that p73-dependent arrest occurs in both G<sub>1</sub> and G<sub>2</sub>-M, similar to that by p53. Although p73 can induce p21, the level of p21 induced by p73 $\alpha$  and p73 $\beta$  in p73 $\alpha$ -22 and p73 $\beta$ -9 cells is six and three times lower than that by p53 in p53-3 cells, respectively (Fig. 3A). Two p53 target genes that can cause growth suppression and may be involved in cell cycle arrest, *i.e.*, *GADD45* (15) and *BTG2* (6), are only weakly activated by p73. Therefore, it remains to determine whether the level of p21 induced by p73 is sufficient to cause cell cycle arrest and whether other cellular genes may also be involved in p73-dependent cell cycle arrest.

It has been shown that p53-dependent G<sub>2</sub>-M arrest is at least, in part, mediated by up-regulation of *14-3-3 $\sigma$*  (5). The gene product interacts with *cdc25* phosphatase to block activation of cyclin B-dependent *cdc2* kinase, which is required for initiation of mitosis, leading to arrest in G<sub>2</sub>-M (16). We found that p73 can also activate *14-3-3 $\sigma$*  (Fig. 3F), suggesting that a signaling pathway to induce arrest in G<sub>2</sub>-M is conserved between p53 and p73. Since activation of *14-3-3 $\sigma$*  by p73 is 3–6 times higher than by p53, it suggests that *14-3-3 $\sigma$*  may be a bona fide cellular target of p73, even though it was originally identified as a potential p53 target gene.

Although both p53 and p73 can induce apoptosis, the signaling pathway leading to apoptosis may differ from each other on the basis

Table 1 Induction of cellular targets by p53 and p73

	Fold increase in relative mRNA <sup>a</sup>			
	p53	p73 $\alpha$	p73 $\beta$	p73 $\alpha$ 292
p21	17.5	3.2	6.4	1.4
MDM2	14.8	1.4	6.5	0.9
GADD45	4.3	1.5	3.6	1.6
BAX	3.6	0.8	1.2	1.0
BTG2	38.9	4.2	10.1	0.8
<i>14-3-3<math>\sigma</math></i>	3.0	17	6.5	ND <sup>b</sup>
p85	2.8	2.0	2.5	1
<i>KILLER/DR5</i>	3	1.2	3	0.7
<i>PIG2</i>	5.0	1.5	0.7	1.0
<i>PIG3</i>	21	2	5	0.5
<i>PIG6</i>	9.9	ND	ND	ND
<i>PIG7</i>	2.4	3.0	5.0	0.9
<i>PIG8</i>	4.7	3	3	0.8
<i>PIG11</i>	15.4	ND	ND	ND
<i>MCG-B61</i>	3.3	0.9	3.0	0.7
<i>MCG-B69</i>	4.3	3.0	3.5	0.7
<i>MCG-B71</i>	3.5	0.7	1.0	1.0

<sup>a</sup> Fold = mRNA(+p53 or +p73)/mRNA(-p53 or -p73).

<sup>b</sup> ND, not done.

of the differential ability of p73 to activate some p53 cellular target genes. *BAX* and several redox-related genes (*PIG2*, *PIG3*, *PIG6*, and *PIG11*), that may be involved in mediating p53-dependent apoptosis (8, 10), were not significantly induced by p73 (Fig. 3). *KILLER/DR5*, a gene that may mediate p53- and DNA damage-induced apoptosis (9), was weakly induced by p53 and p73 $\beta$  but not p73 $\alpha$  in H1299 cells (Fig. 3). Since we have shown that p73 $\alpha$  is nearly as active as p73 $\beta$  in inducing apoptosis (Fig. 2), the results suggest that *KILLER/DR5* may not be involved in p73-dependent apoptosis unless an apoptotic signaling pathway for p73 $\alpha$  is different from that for p73 $\beta$ . Although *PIG7*, *PIG8*, and *p85* were significantly induced by p73, the activity of *PIG7* and *PIG8* in apoptosis is still unknown, and the role of *p85* in apoptosis seems to be restricted to cellular response to oxidative stress (11). Because the transactivation-deficient p73 $\alpha$ 292 is incapable of inducing apoptosis (Refs. 2 and 3; present study), we hypothesize that a distinctive group of cellular genes that can be activated by p73 may be responsible for mediating apoptosis. Therefore, identification of such p73 target genes is necessary to understand the mechanism of p73-dependent apoptosis.

*MDM2*, an oncogene that negatively regulates p53 activity (14), was weakly induced by p73 $\beta$  but not p73 $\alpha$  (Fig. 3B). It has been shown that a physical interaction between p53 and *MDM2* enhances degradation of p53 through the ubiquitination pathway (17, 18) as well as conceals the activation domain of p53 to regulate transcription (19). Although it is not clear whether p73 physically interacts with *MDM2*, failure to activate *MDM2* by p73 $\alpha$  suggests that the activity of p73 $\alpha$  is not subject to regulation by *MDM2*. Thus, in addition to a previous report that p73 is not responsive to DNA damage (2), it is also regulated differently from p53 by *MDM2*.

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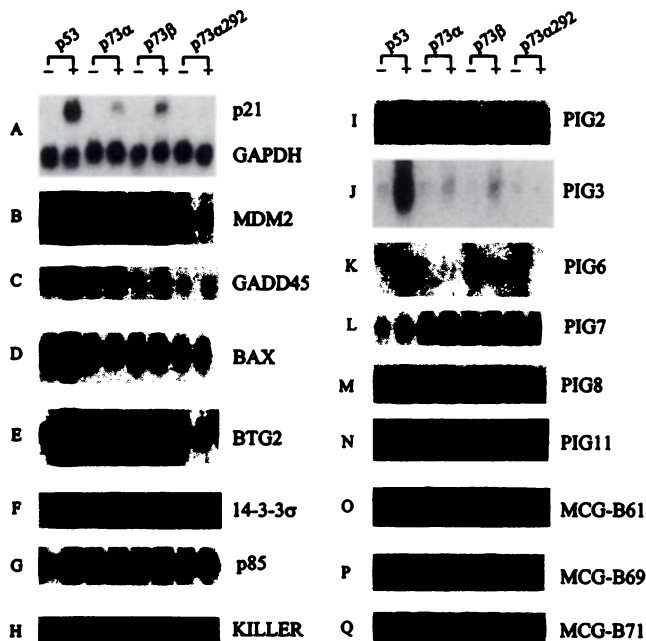


Fig. 3. p73 differentially activates some but not all of the p53 cellular target genes. Northern blots were prepared using 10  $\mu$ g of total RNA isolated from p53-3, p73 $\alpha$ -22, p73 $\beta$ -9, or p73 $\alpha$ 292-20 cells under both the uninduced (-) and induced (+) conditions. The blots were probed with cDNAs corresponding to the genes as indicated on the right side of the blots.

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