*Advances in Brief*

# p73 Is Effective in p53-null Pancreatic Cancer Cells Resistant to Wild-type *TP53* Gene Replacement[1]

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## Abstract

Novel therapies such as gene therapy are needed for the treatment of pancreatic carcinomas. Here we show that adenovirus-mediated p73 overexpression results in a strong induction of apoptosis, whereas the effect of p53 varies between different cell lines. In particular, p53-negative AsPC-1 cells are resistant to p53-mediated apoptosis. In these cells, only ectopically expressed p73 activates the proapoptotic p53 target *P53AIP1*, whereas phosphorylation of p53 at Ser-46, shown to regulate transcriptional activation of *P53AIP1*, is missing. Our findings support the use of p73 as an anticancer drug in p53-null pancreatic cancer cells that are resistant to wild-type *TP53* gene replacement.

## Introduction

Pancreatic cancer represents one of the most common causes of cancer-related death in industrialized Western countries (1). At present, no curative therapeutic approaches are available for the disease, and the 5-year survival rate is the lowest of any type of cancer (2). Alternative treatment modalities for the treatment of pancreatic cancer are therefore urgently needed.

Inactivation of the p53 tumor suppressor appears to be an essential step in human tumorigenesis (3). This is clarified by the fact that mutations within the *TP53* gene are among the most frequently detected alterations in human carcinomas (4). Mutations of *TP53* occur in up to 70% of pancreatic adenocarcinomas. The lack of functional p53 has been proposed to be a component of resistance to DNA-damaging agents, resulting in the inhibition of apoptosis (5). Thus, reintroduction of wt[3] p53 is a commonly used gene therapeutic strategy for the treatment of various types of cancer, including pancreatic cancer (6).

However, induction of apoptosis by the wt p53 protein has not been realized in all cases due to the resistance of some tumors to exogenous p53 (7). To overcome these restrictions, genes that promote apoptosis by p53-independent mechanisms are particularly useful. The recently identified p53 family member, p73, represents such a molecule. When overexpressed, p73 binds to p53 DNA target sites, transactivates p53-responsive genes, and is capable of inducing cell cycle arrest and apoptosis in mammalian cells in a p53-like manner (8). Several studies have shown that the β-isoform of p73 is more potent in transactivation and more effective in inducing apoptosis than the α-isoform (9).

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[3] The abbreviations used are: wt, wild-type; Ad, adenovirus; GFP, green fluorescent protein; CMV, cytomegalovirus; RT-PCR, reverse transcription-PCR; MOI, multiplicity of infection; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; AS, antisense; SE, sense.

In this study, we analyzed the apoptotic activity of p73 compared with p53 in a variety of human pancreatic cancer cell lines lacking functional p53. Our data indicate that p73β is capable of efficient killing of cells that are completely resistant to p53-mediated apoptosis. Moreover, we show that the loss of p53 proapoptotic activity in these cells is due to the lack of p53 phosphorylation at Ser-46 shown to be required for transcriptional activation of *P53AIP1*.

## Materials and Methods

**Cell Culture.** Human pancreatic adenocarcinoma cell lines AsPC-1 (ATCC CRL 1682), Capan-1 (ATCC HTB 79), Capan-2 (ATCC HTB 80), and MZA (obtained from D. I. Smith, Mayo Clinic, Rochester, MN) were maintained in DMEM supplemented with 10% FCS. H1299 cells (human lung carcinoma) were also grown in DMEM supplemented with 10% FCS. Viruses were grown in 293 (Ad5 E1-transformed human embryonic kidney cells) cells maintained in modified Eagle's medium F-11 with 10% fetal bovine serum. Media were supplemented with 2 mM L-glutamine, 100 μg/ml penicillin, and 100 units/ml streptomycin.

**Construction of Adenoviral Vectors.** All adenoviral vectors were generated using the AdEasy System (Ref. 10; kindly provided by B. Vogelstein). Adp73 expressing the p73 β-isoform was constructed as follows: the p73β cDNA was isolated from the pcDNA3.1 plasmid (a kind gift of G. Melino) by *Xho*I/*Kpn*I digestion and cloned into pShuttle-CMV plasmid under the control of the CMV promoter terminated by the SV40 polyadenylation signal, resulting in pShuttle-CMV-p73β. Virus was generated by homologous recombination after cotransformation with pAdEasy1 in *Escherichia coli* BJ5183. The vectors AdGFPS and Adp53 have been described previously (11). All viruses were propagated, purified, and titrated as described. Adenoviral infections were carried out at MOIs that allow 100% transduction of each cell line (MOI = 10 for MZA and H1299 cells, 30 for AsPC-1 cells, 100 for Capan-1 cells, and 300 for Capan-2 cells).

**Flow Cytometry and MTT Assay.** For flow cytometry analysis, cells were seeded on 60-mm dishes. At 60–80% confluence, cells were infected with Ad. Cells were harvested 48 h after infection, fixed in 70% ethanol, and stained for DNA content with propidium iodide. Flow cytometric analysis was carried out (FACSVantage; Becton Dickinson) and analyzed as described previously using CellQuest software (Becton Dickinson; Ref. 12). For MTT assay, cells were seeded on 96-well plates and infected with Ad-vector. Triplicate wells were assayed every 24 h for cell viability using the CellTiter96 AQ$_{ueous}$ One Solution Cell Proliferation Assay (Promega, Mannheim, Germany).

**Semiquantitative RT-PCR and Western Blotting.** RT-PCR was performed on total RNA prepared by RNeasy Mini Kit (Qiagen, Hilden, Germany) as described previously (12). The upstream and downstream primers used were as follows: (*a*) for *CDKN1A/p21*, 5′-TTCTAATGCCAGAG-GCTGG-3′ and 5′-TGTGGACCTGTCACTGTCTTG-3′; (*b*) for *MDM2*, 5′-TGCTACTAGAAGTTGATGGCTGAG-3′ and 5′-TCAAGTTACTGTGT-ATCAGGCAGG-3′; (*c*) for *PIDD*, 5′-GCACCAGGCAGGCATTGGAC-3′ and 5′-GCCATGTGCTGGAGCTGCAG-3′; (*d*) for *PIG3*, 5′-TTGCTGGC-TCCTGGAGGTGG-3′ and 5′-TGGCTGCTCCGCGAGGATAC-3′; (*e*) for *BAX*, 5′-CCCCAGTTGAAGTTGCCGTC-3′ and 5′-GATCATGAAGA-CAGGGGCCC-3′; (*f*) for *P53AIP1*, 5′-TGGCTCCAGGAAGGAAAGG-C-3′ and 5′-TGCTTTCTGCAGACAGGGC-3′; and (*g*) for *GAPDH*, 5′-CACAGTCCATGCCATCAC-3′ and 5′-CACCACCCTGTTGCTGTA-3′. For Western blot analysis, cell lysates were prepared after infection, and protein levels were analyzed essentially as described previously (12) using monoclonal antihuman p53 (Ab-6; Calbiochem, Bad Soden, Germany), anti-
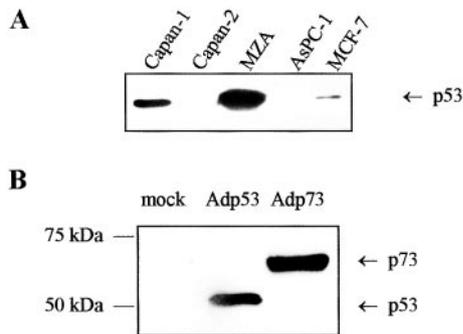
Fig. 1. Efficacy of Ad vector-mediated gene transfer in p53-null human pancreatic cancer cell lines. *A,* Western blot of endogenous p53 expression. MCF-7 cells were used as control (wt p53). *B,* Western blot of p53 and p73 in AsPC-1 cells 24 h after infection with Adp53 or Adp73. Mock-transfected cells are shown as a control.

cells were analyzed by fluorescence-activated cell-sorting analysis 48 h after treatment.

**Immunfluorescence Microscopy.** Cells were seeded on glass coverslips and infected with Adp53. Twenty-four h after infection, cells were fixed with ice-cold methanol/acetone (1:1). The slides were washed twice with PBS, blocked with 5% BSA in PBS for 20 min, and incubated with primary antibody (anti-p53 Ab-6; Calbiochem) for 1 h. After washing with PBS, the slides were incubated with the appropriate secondary antibody (Alexa Fluor[546]-conjugated antimouse antibody; Molecular Probes, Leiden, the Netherlands) and 4′,6-diamidino-2-phenylindole (10 $\mu$g/ml) for 45 min and subjected to fluorescence microscopy or fluorescence-activated laser scanning microscopy.

**Xenograft Assay.** AsPC-1 cells ($1 \times 10^7$) infected with Adp53, Adp73, or AdGFPS were injected s.c. into the right flank of 6–8-week-old *nu/nu* (nude) mice. Mice were monitored for tumor growth at weekly intervals, and tumors were measured in two perpendicular diameters using calipers. Animal work has been approved by and carried out according to guidelines set by the University Institutional Animal Care and Use Committee.

## Results and Discussion

**Efficacy of p53 and p73 in Pancreatic Carcinoma Cell Lines.** Several previous reports have indicated that the expression of wt *TP53* gene in p53-null carcinoma cells does not always produce the therapeutic effects attributed to p53 (9, 13, 14). Of the human pancreatic cancer cell lines used in this study AsPC-1 and Capan-2 cells are p53 negative, whereas Capan-1 (15) and MZA (point mutation R248W; data not shown) cells express mutated p53 protein (Fig. 1*A*). The transduction efficiency of Ad in the various pancreatic cancer cell

human phospho-Ser-15 p53 (9284; Cell Signaling), antihuman phospho-Ser-46 p53 (2521; Cell Signaling), or antihuman p73 antibody (ER-15; PharMingen). Antibody binding sites were visualized by enhanced chemiluminescence (Amersham Pharmacia Biotech, Braunschweig, Germany).

**AS Oligonucleotides.** To inhibit expression of endogenous p53AIP1, high-performance liquid chromatography-purified AS oligonucleotide was prepared (TCCCCTGGATGGGATC). The SE oligonucleotide (GATCCCATC-CAGGGGA) was used as a control. Oligonucleotides (1 $\mu$M) were transfected with Oligofectamine reagent (Life Technologies, Inc.). At 4 h after transfection, cells were infected with either Adp73 or the control vector. Apoptotic



Fig. 2. Analysis of cell killing in pancreatic cancer cells. *A,* cell viability of MZA and AsPC-1 cells over 3 days after Adp53 (▨) or Adp73 (▯) treatment, as measured by MTT assay. Cell viability of control vector-treated cells (■) was set as 100%. The number of viable cells is the mean ± SD of three different wells; *bars,* SD. Triplicate experiments were performed for each cell line. *B, left panels,* flow cytometry DNA profiles of Capan-1, Capan-2, MZA, and AsPC-1 cells 48 h after infection with AdGFPS (*GFP*), Adp53 (*p53*), and Adp73 (*p73*). Apoptotic cell population is labeled as M1. *Right panel,* level of apoptosis is as indicated (−, 0–10% sub-G$_1$; +, 10–20% sub-G$_1$; ++, 20–40% sub-G$_1$; +++, >40% sub-G$_1$). *C,* xenograft assay of AsPC-1 cells infected with Ad vector expressing p53, p73, or GFP. Tumor volume was measured over 28 days after injection. ●, GFP (*n* = 5); ▲, p53 (*n* = 5); ■, p73 (*n* = 5).
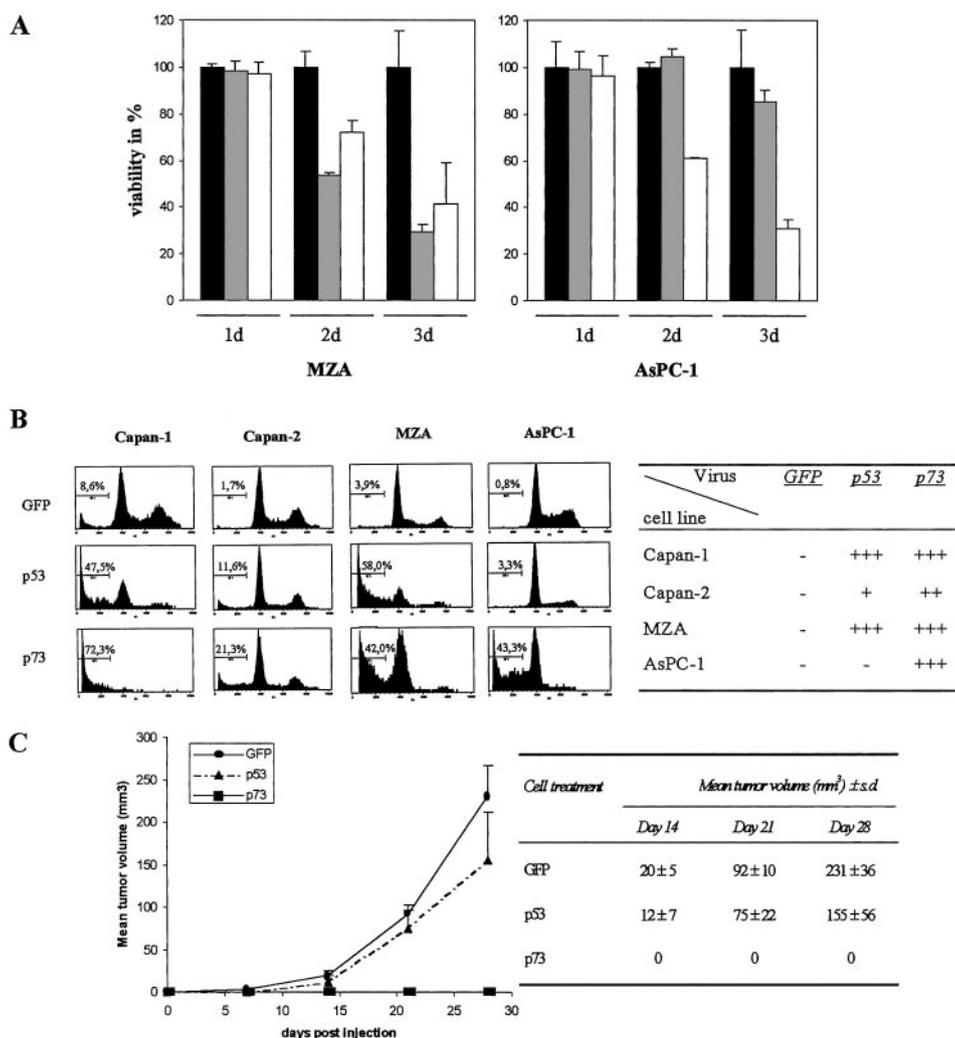
lines has been evaluated previously (12). To assess the effect of Ad vector-mediated expression of p53 and the β-isoform of p73, which was shown to be more effective in inducing apoptosis than p73α (9), pancreatic cells were infected with Adp53 and Adp73, respectively, at a multiplicity of infection that allows 100% transduction. As shown in Fig. 1B, strong protein expression was evident at 24 h after infection in AsPC-1 cells.

Next, we evaluated whether p53 or p73 affects the viability of pancreatic tumor cells. Compared with control vector-infected cells, we observed substantial differences in cell viability by overexpression of wt p53 between the various pancreatic cell lines (shown for MZA and AsPC-1 in Fig. 2A) with apoptotic rates ranging from 3% in AsPC-1 to 58% in MZA cells (Fig. 2B). Whereas wt *TP53* gene transfer led to a significant increase in the sub-$G_1$ population of MZA and Capan-1 cells, as shown by flow cytometry (Fig. 2B), p53-mediated cytotoxicity in Capan-2 cells was only moderate (11.6%; Fig. 2B). Importantly, p53 overexpression had no cell killing effect in AsPC-1 cells (Fig. 2, A and B). In contrast to p53, Ad-vector-mediated expression of p73 resulted in a profound loss of cell viability in all pancreatic cancer cells (Fig. 2, A and B). As shown in Fig. 2B, significant amounts of apoptotic sub-$G_1$ cells were detectable at 48 h after infection with apoptotic rates between 21.3% in Capan-2 cells and 72.3% in Capan-1 cells. In contrast to p53, ectopic expression of

p73 in AsPC-1 cells resulted in massive cell killing (43.3%; Fig. 2B). The difference in p73 *versus* p53 activity in AsPC-1 cells shown in the short-term assays has also been confirmed in a xenograft assay, where AsPC-1 cells infected with Adp53, Adp73, or the AdGFPS control virus were injected into nude mice and subsequently monitored for tumor growth over 4 weeks. Whereas injection of tumor cells expressing p53 resulted in sustained tumor growth in all animals similar to control vector-injected cells, a significant lack of tumor formation was evident in mice that received p73-expressing AsPC-1 cells (Fig. 2C). These data indicate that p73 is able to induce apoptosis in p53-null cells that are resistant to wt p53.

**Analysis of p53 and p73 Function in AsPC-1 Cells.** In addition to our findings, other recent studies reported on the resistance of AsPC-1 cells to p53-mediated apoptosis (13, 16), but the underlying mechanism is unknown. Therefore, we next investigated why *TP53* gene replacement in p53-negative AsPC-1 cells did not lead to the induction of apoptosis. Possible mechanisms for the lack of p53 activity in these cells might rely on protein degradation shortly after expression or on the retention of the p53 transcription factor in the cytoplasm. After infection of AsPC-1 cells with Adp53, expression of the p53 protein was analyzed over a longer time period sufficient to induce cytotoxic effects in these cells. Western blot analysis revealed stable p53 levels over at least 3 days with no sign of degradation (Fig.
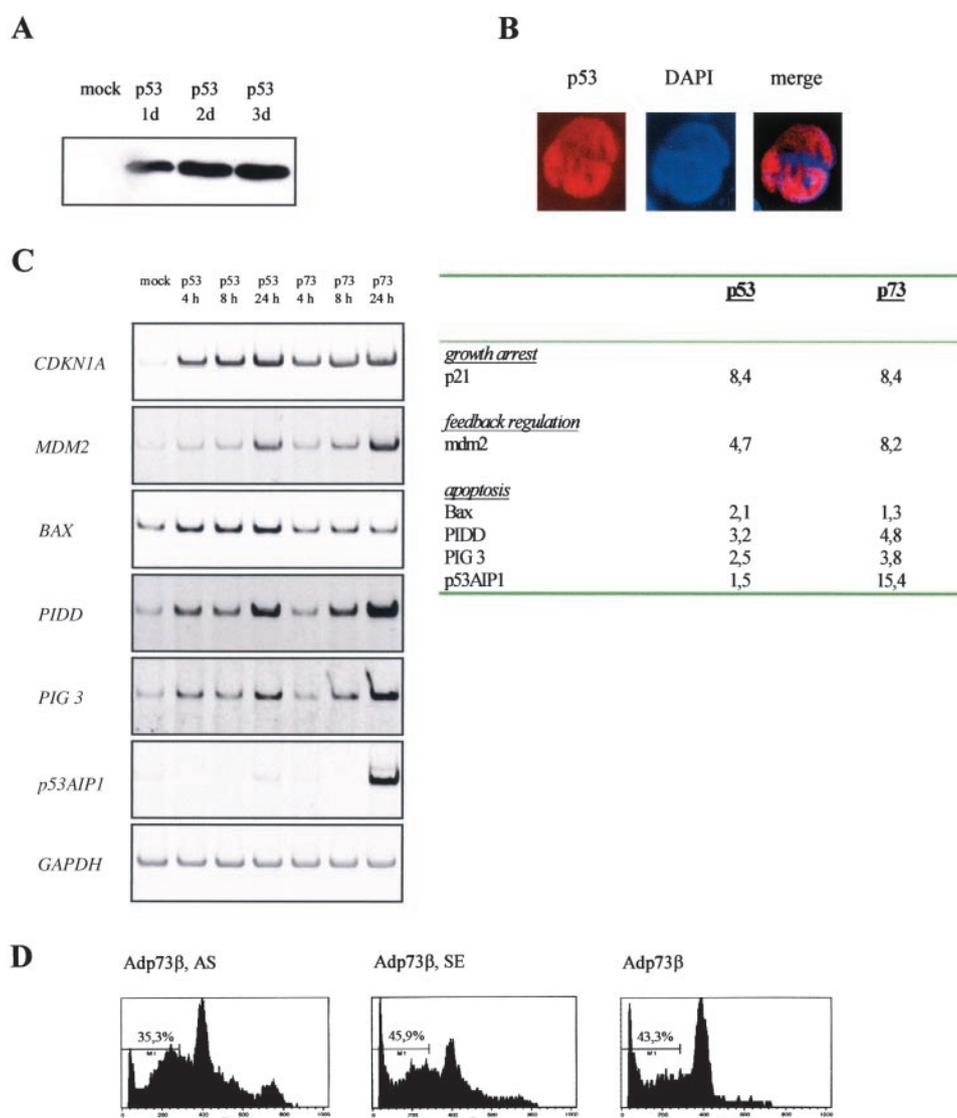
Fig. 3. Analysis of p53 function in AsPC-1 cells. *A,* Western blot of p53 at 24, 48, and 72 h after Adp53 treatment. Mock transfection is shown as a control. *B,* representative images of AsPC-1 cells stained for exogenous p53 by indirect immunofluorescence 24 h after infection with Adp53 (*p53*). Nuclear DNA was stained with 4′,6-diamidino-2-phenylindole (*DAPI*). Overlapping localization is shown in the third panel (*merge*). *C,* RT-PCR analysis of expression of target genes. *Left panels,* RT-PCR of AsPC-1 cells infected with Adp53 (*p53*) or Adp73β (*p73*) at the indicated time points. *GAPDH* is shown as a control. *Right panel,* induction of target genes by p53 and p73. PCR products were quantitated in relative software units by the Bio-Imaging Analyzer (Fuji) using TINA program version 2.09 (shown as fold induction). The data were normalized to *GAPDH* values, and the untreated control was set as 1. *D,* effect of p53AIP1 inhibition on p73-dependent apoptosis. AS or SE oligonucleotides were transfected into AsPC-1 cells for 4 h and subsequently infected with Adp73. Apoptotic cells were evaluated by fluorescence-activated cell-sorting analysis after 48 h.

| | p53 | p73 |
|---|---|---|
| *growth arrest* | | |
| p21 | 8,4 | 8,4 |
| *feedback regulation* | | |
| mdm2 | 4,7 | 8,2 |
| *apoptosis* | | |
| Bax | 2,1 | 1,3 |
| PIDD | 3,2 | 4,8 |
| PIG 3 | 2,5 | 3,8 |
| p53AIP1 | 1,5 | 15,4 |

## H1299

mock  p53    p53    p53    p53
      18h    21h    24h    40h

## AsPC-1

mock  p53    p53    p53    p53
      18h    21h    24h    40h
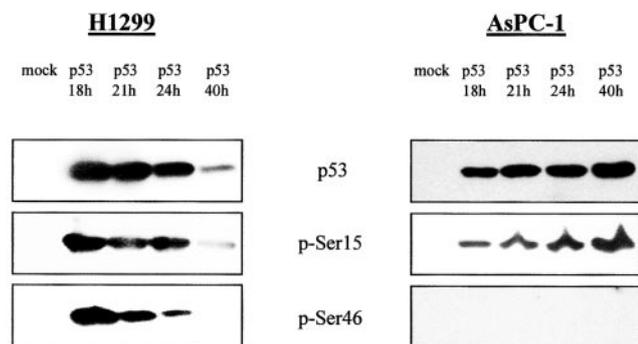


p53

p-Ser15

p-Ser46

Fig. 4. Analysis of p53 phosphorylation at Ser-46 in AsPC-1 cells. Western blot analysis of AsPC-1 and H1299 cells (positive control) infected with Adp53 at the indicated times. Phosphorylation of p53 at Ser-15 and Ser-46 was detected by using specific antibodies (see "Materials and Methods").

*3A*). Cellular localization of ectopically expressed p53 protein was visualized by immunofluorescence and confocal laser microscopy. As shown in Fig. 3*B*, exogenous p53 protein became correctly translocated to the nucleus of AsPC-1 cells. However, it is known that p53 inhibits cell growth through its binding to specific DNA sequences, which leads to the transactivation of target genes involved in cell cycle control (*e.g., CDKN1A/p21* and *MDM2*) and/or apoptosis [*e.g., BAX, PIDD, PIG3,* and *P53AIP1* (3, 17)]. When overexpressed, p73 also binds to p53 DNA target sites and transactivates p53-responsive genes (8). To investigate whether differences in the expression of downstream targets account for the functional discrepancy between p53 and p73 in AsPC-1 cells, RT-PCR analysis of Adp53- and Adp73-infected cells was performed. As shown in Fig. 3*C*, transduction of both p53 and p73 markedly increased the expression levels of *CDKN1A/p21* (8.4-fold), *MDM2* (4.7- and 8.2-fold), *PIDD* (3.2- and 4.8-fold), and *PIG3* (2.5- and 3.8-fold). Whereas p53 led to a 2.1-fold up-regulation of *BAX*, no significant increase of the p53 target gene occurred by p73 expression. Interestingly, *P53AIP1*, an important mediator of p53-dependent apoptosis (18), was strongly activated by p73 with a 15.4-fold induction compared with the only 1.5-fold increase observed for p53 (Fig. 3*C*).

To see whether up-regulation of endogenous p53AIP1 protein is involved in p73-mediated apoptosis in AsPC-1 cells, *P53AIP1* expression was inhibited by using a specific p53AIP1 AS oligonucleotide. As shown in Fig. 3*D*, treatment of AsPC-1 cells with AS oligonucleotide resulted in a significant reduction of apoptotic cells to a level approximately 23% of that seen in Adp73-infected cells alone or in the presence of SE oligonucleotide. This experiment clearly shows that Adp73-mediated cell death in AsPC-1 cells is due, at least in part, to the activation of p53AIP1.

**Lack of p53 Phosphorylation at Ser-46 Prevents Activation of p53AIP1 in AsPC-1 Cells.** p53 is extensively phosphorylated, and modification at several residues has been specifically associated with the ability of p53 to induce an apoptotic response. Of particular interest is the phosphorylation of p53 on Ser-46. A recent study revealed that transcriptional activation of *P53AIP1* by p53 is regulated by phosphorylation of p53 at serine residue 46 in H1299 cells (18). To determine whether exogenous p53 protein is phosphorylated at Ser-46 in AsPC-1 cells, cells were infected with Adp53, and p53 phosphorylation was detected by Western blot analysis using antihuman phospho-Ser-46 p53 antibody (Fig. 4*A*). High amounts of p53 protein were expressed in both p53-negative AsPC-1 and H1299 cells (used as a positive control) after Adp53 infection. In addition, phosphorylation of p53 at Ser-15, shown to be involved in activating p53 (19), was detectable in both cell lines (Fig. 4, *p-Ser15*). In contrast, p53 protein phosphorylated at Ser-46 could only be detected in H1299

cells, whereas AsPC-1 cells infected with Adp53 revealed no signal (Fig. 4, *p-Ser46*), indicating that ectopically expressed p53 is not phosphorylated at Ser-46 in these cells. These data imply that the loss of p53 proapoptotic activity in AsPC-1 cells might be due to the lack of p53 phosphorylation at Ser-46.

Thus far, there is accumulated evidence that phosphorylation of Ser-46 is essential in regulating the ability of p53 to induce expression of apoptotic target genes, and several kinases have been implicated as being responsible for this modification. It has been shown that the homeodomain-interacting protein kinase 2 and the p38 mitogen-activated protein kinase mediate Ser-46 phosphorylation in response to UV irradiation. Beside the activation of kinases, expression of cofactors for the putative p53 Ser-46 kinase (*e.g.,* p53-dependent damage-inducible nuclear protein 1, p53DINP1) has been shown to regulate p53-dependent apoptosis through phosphorylation of Ser-46. In addition, repression of phosphatases such as WIP1, which inactivates p38 mitogen-activated protein kinase, is involved in the regulation of Ser-46 phosphorylation (17). Therefore, it is possible that the lack of p53 phosphorylation on Ser-46 in AsPC-1 cells relies on the absence of these kinases or kinase cofactors. However, with respect to p73 activity in AsPC-1 cells, it is very likely that the mechanism of p53 activation through phosphorylation is dispensable for p73. This is supported by previous findings indicating that both p53 and p73 become phosphorylated in response to DNA damage, but selective activation of proapoptotic target genes such as *P53AIP1* by p73 resulting in apoptosis induction is dependent on p73 acetylation (20).

Together, our results strongly support the use of p73β as a beneficial approach to overcome the resistance of p53-null pancreatic carcinoma cells to wt *TP53* gene transfer. In this context, a previous study by Prabhu *et al.* (21) has pointed to the advantage of p73β over p53 in cervical cancer cells carrying the human papillomavirus E6 transgene that leads to p53 degradation, whereas p73β is resistant to E6-mediated proteolysis. Furthermore, it has recently been demonstrated that colorectal cancer cell lines that are resistant to p53-mediated cell death undergo apoptosis after transduction of p73β (9), suggesting that differences in apoptotic effects of p53 or p73 overexpression reflect the genetic characteristics of the recipient cancer cells. Whether impaired p53 phosphorylation is the mechanism underlying the different responses between both p53 family members in other cell lines has to be clarified.

### Acknowledgments

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