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Research article

THE CELL TYPE-SPECIFIC EFFECT OF TAp73 ISOFORMS ON THE CELL CYCLE AND APOPTOSIS

JITKA HOLCAKOVA[1#], PAVLA CESKOVA[1#], ROMAN HRSTKA[1], PETR MULLER[1], LENKA DUBSKA[2], PHILIP J. COATES[3], EMIL PALECEK[4] and BORIVOJ VOJTESEK[1]*

[1]Department of Oncological and Experimental Pathology, Masaryk Memorial Cancer Institute, Zluty kopec 7, 656 53 Brno, Czech Republic, [2]Department of Laboratory Medicine, Masaryk Memorial Cancer Institute, Zluty kopec 7, 656 53 Brno, Czech Republic, [3]Division of Pathology and Neurosciences, Ninewells Hospital and Medical School, University of Dundee, DD1 9SY Dundee, UK, [4]Institute of Biophysics AS CR, Kralovopolska 135, 612 65 Brno, Czech Republic

Abstract: p73, a member of the p53 family, exhibits activities similar to those of p53, including the ability to induce growth arrest and apoptosis. p73 influences chemotherapeutic responses in human cancer patients, in association with p53. Alternative splicing of the *TP73* gene produces many p73 C- and N-terminal isoforms, which vary in their transcriptional activity towards p53-responsive promoters. In this paper, we show that the C-terminal spliced isoforms of the p73 protein differ in their DNA-binding capacity, but this is not an accurate predictor of transcriptional activity. In different p53-null cell lines, p73β induces either mitochondrial-associated or death receptor-mediated apoptosis, and these

* Author for correspondence; e-mail: vojtesek@mou.cz, tel.: +420 543133303

[#] These authors contributed equally to this paper

Abbreviations used: ADO22 – TRAF and TNF receptor-associated protein-22; APEX – apurinic/apyrimidinic endonuclease 1/redox factor; ASPP2 – apoptosis stimulating proteins of p53; CDK1(cdc2) – cyclin-dependent protein kinase-1; D-MEM – Dulbecco's modified Eagle medium; DR5 – trail death receptor; EMSA – electrophoretic mobility-shift assay; FAF1 – Fas-associated factor; FBS – foetal bovine serum; IVTT – *in vitro* transcription/translation; MAP4 – microtubule-associated protein 4; PIDD – p53-induced protein with a death domain; PML – promyelocytic leukaemia gene; SDS-PAGE – SDS-polyacrylamide gel electrophoresis; TRAFs – TNF receptor-associated factor

differences are reflected in different gene expression profiles. In addition, p73 induces cell cycle arrest and $p21^{WAF1}$ expression in H1299 cells, but not in Saos-2. This data shows that TAp73 isoforms act differently depending on the tumour cell background, and have important implications for p73-mediated therapeutic responses in individual human cancer patients.

Key words: p53, TAp73, ΔTAp73, DNA binding, Transactivation, Cell cycle, Apoptosis

INTRODUCTION

TP73 is a family member of the *TP53* tumour suppressor gene family. *TP53* and *TP73* share significant structural and functional homology, and both induce cell cycle arrest and cell death in response to DNA damage. Like p53, p73 can activate the promoters of several p53-responsive genes, including $p21^{WAF1}$, *bax*, *mdm2*, *cyclin-G*, *gadd45* and *IGFBP3*. It was also shown that many target genes respond differently to p53 or p73 proteins. Cancer-related alterations of the *TP73* gene include changes in alternative splicing at the C-terminus and the generation of TAp73 or ΔTAp73 N-terminal isoforms [1-6]. p73α and p73β are the major products of 3' alternative splicing, with p73β being more effective for transcriptional activation than p73α. The protein products of 5' alternative splicing (ΔTAp73) have very low or no transactivation activities. ΔTAp73 isoforms inhibit wild-type p53 and TAp73 by either competing for binding to the promoters of p53-responsive genes, or by making transactivation deficient hetero-oligomers with p53 or TAp73. This data suggests that *TP73* encodes a putative tumour suppressor protein (TAp73) and a putative oncoprotein (ΔTAp73). By extension, tumour cells may compensate for the function of mutant p53 protein by expressing transcription-deficient p73 isoforms, which inhibit the wild-type p53 and TAp73 [7].

As a similarity was found between the abilities of the p53 family of genes and of p73 to transactivate p53 target genes, their roles in DNA damage-induced apoptosis was explored. A combined loss of p63 and p73 resulted in the failure of cells containing functional p53 to undergo apoptosis in response to DNA damage [8]. Blocking p73 function with a dominant-negative mutant, siRNA, or homologous recombination led to the chemoresistance of human tumour cells and the development of transformed cells, irrespective of p53 status. Mutant p53 inactivates p73, and down-regulation of mutant p53 enhances chemosensitivity [9]. Similarly, endogenous expression of ΔNp63 in squamous carcinoma cells enhances their survival by inhibiting TAp73 activity [10].

Given the importance of p73 in human cancer, it is necessary to understand the precise role(s) of the p73 protein isoforms, including their abilities to transcriptionally activate specific target genes that function in carcinogenesis or in the apoptotic response to genotoxic tumour therapies. We analyzed the *in vitro* binding activity of different TAp73 C-terminal splicing isoforms to

p53-responsive elements, and characterised their *in vivo* transcriptional activity. We also compared their *in vitro* binding activity with their ability to induce apoptosis *in vivo*.

MATERIAL AND METHODS

Plasmids and oligonucleotides

The plasmids pcDNA3-HA-TAp73α, β, γ, δ (provided by G. Melino, [3]), and pcDNA3-wild-type p53 were used for eukaryotic transfection and *in vitro* transcription/translation. The plasmids rasadluc2, baxluc and pmdm2luc, which are the reporter constructs of luciferase cDNA under the control of a promoter respectively containing the p53-responsive elements *ras*, *bax* and *mdm2*, were used for co-transfection experiments. The plasmid pBluescript served as a negative control.

The following oligonucleotides containing single p53 recognition sequences were used:

CON: AGACATGCCTAGACATGCCT
$p21^{WAF1}$: GAACATGTCCCAACATGTTG
gadd45: GAACATGTCTAAGCATGCTG
bax: TCACAAGTTAGAGACAAGCCTGGGCGTGGGCTATATT
mdm2: GGTCAAGTTGGGACACGTCC
ras: GGACATGCCCGGGCATGTCC
RGC: TGCCTTGCCT GGACTTGCCT GGCCTTGCCT
mutated $p21^{WAF1}$: GAA<u>A</u>AT<u>T</u>TCCCAACATGTTG

The complementary oligonucleotides were hybridised and end-labeled with [γ-^{32}P] ATP.

Antibodies

The antibodies used were: DO-1 monoclonal antibody recognizing human p53 [11], 2A9 monoclonal antibody recognizing MDM-2 [12], 118 monoclonal antibody recognizing $p21^{WAF1}$ [13], AC-40 recognizing beta-actin (Sigma-Aldrich Inc., USA), and Anti-HA-tag (Santa Cruz Biotechnology Inc., USA). The antibody concentrations used for western blotting were 1 μg/ml, and for the gel shift assay, 200 ng of antibody per reaction.

In vitro transcription/translation (IVTT)

Plasmids carrying the cDNA for the HA-tagged TAp73α, TAp73β, TAp73γ and TAp73δ proteins were translated using the TNT Quick Coupled Transcription/Translation System (Promega Corp., USA). The amount of the protein was assessed using SDS-PAGE, Western blotting and immunodetection with anti-HA antibody.

SDS-PAGE and immunoblotting

In vitro translated proteins and cell extracts were lysed directly in Laemmli sample buffer. 20 μg was separated by SDS-polyacrylamide gel electrophoresis

(SDS-PAGE) on 10% or 12.5% gels and transferred onto nitrocellulose membranes. The membranes were blocked in 5% milk and 0.1% Tween 20 in PBS for 2 h at room temperature, and probed overnight with specific monoclonal antibodies. To confirm equal protein loading, immunodetection was performed with AC-40. Peroxidase-conjugated rabbit anti-mouse immunoglobulin antiserum (DAKO Ltd., Denmark) diluted 1:1000 was used as the secondary antibody. The visualization was done with ECL reagents from Amersham-Pharmacia (UK).

Electrophoretic mobility-shift assay (EMSA)

IVTT proteins (2 μl) or 100 ng of p53 protein expressed in Sf9 cells with or without 200 ng of the specific monoclonal antibody was added to 1 ng of radiolabeled double-stranded oligonucleotides and 20 ng of competitor DNA (pBluescript/*Sma*I) in a reaction buffer (25 mM Tris pH 7.6, 20% glycerol, 1 mg/ml BSA, 5 mM DTT, 50 mM KCl, 0.1% Triton X-100) and incubated at room temperature for 30 min. The samples were analysed via native polyacrylamide gel electrophoresis in 4% gels in 0.33 x TBE buffer (89 mM Tris-borate, 2 mM EDTA) at 200 V for 120 min at 4ºC. Binding was quantified densitometrically after radiographic detection. The densitometric intensity of the band corresponding to the p53/oligonucleotide complex was set as 100%. For each TAp73 isoform, the ratio of densitometrical intensity for this isoform compared to p53 protein was calculated, and three parallel experiments were performed. The mean value of the densitometrical intensity for TAp73 isoforms is depicted in the graphs.

Cell culture and transfection

Three cell lines lacking *TP53* – Saos-2 (osteosarcoma cells) [14], H1299 (lung cancer cells) [15] and H1299-RGC (lung cancer cells) – were stably transfected with the β-galactosidase p53-responsive reporter construct (pRGCΔfosLacZ), and were maintained in Dulbecco's modified Eagle medium (D-MEM) supplemented with 10% foetal bovine serum (FBS) in 5% CO_2 at 37ºC, and transfected with individual plasmids containing HA-tagged TAp73 isoforms, p53, or pBlueScript using Lipofectamine (Invitrogen Corp., USA). The status of the p53, p63 and TAp73 isoforms expressed in the Saos-2 and H1299 cell lines is summarized in Tab. 1.

Transactivation of target genes

Transfected H1299-RGC cells were cultured for 24 h, and then washed with PBS, lysed in 0.25 M Tris pH 7.5 by two freeze-thaw cycles, and subjected to double two-second ultrasound pulses at maximum intensity. The lysates were cleared by centrifugation at 14000 rpm for 30 min at 4ºC. The protein concentrations were measured using the Bio-Rad Protein Assay (Bio-Rad Laboratories Inc., USA), and the β-galactosidase activity was assessed with ONPG as a substrate and absorbance at 420 nm [16]. The experiments were performed in triplicate, and the mean value per mg of protein is depicted in the graphs.

Tab. 1. The status of the p53, p63 and p73 mRNA in the H1299 and Saos-2 cell lines.

			Saos-2	H1299
p53	RNA status	wild-type	-	-
	Protein status	MAb DO-1	-	-
p63	RNA status	Alpha	-	+
		Beta	-	-
		Gamma	-	+
		TA	-	+
		Delta	-	-
p73	RNA status	Alpha	-	+
		Beta	-	(+)
		Gamma	-	+
		Epsilon	(+)	(+)
		TA	(+)	+
		Delta	-	+

+ Gave a CT of 20-31 cycles after the amplification of 0.67 μl of cDNA, obtained from a cDNA synthesis kit (Invitrogen Corp, USA, #12328-040) using 1 μg RNA. (+) Gave a CT of 31-34 cycles after the amplification of 0.67 μl of cDNA, obtained from a cDNA synthesis kit (Invitrogen Corp., USA, #12328-040) using 1 μg RNA. - Not detectable or a very high cycle number

Apoptosis and cell cycle determination

To detect the early stage of apoptosis, the expression of the apoptosis-specific mitochondrial antigen 7A6 was examined with Apo2.7 monoclonal antibody as follows. Cells were fixed with 0.25% paraformaldehyde in PBS for 2 min on ice, and washed twice with PBS. Then, 10 mg/ml of digitonin (Sigma-Aldrich Inc., USA) was added for permeabilization, and the cells were labeled with PE-conjugated Apo2.7 monoclonal antibody (Beckman Coulter, Inc., USA) for 30 min on ice. After a wash with PBS containing 0.05% Tween 20, the cells were re-suspended in PBS for analysis by flow cytometer (Beckman Coulter FC500). Early apoptotic cells were Apo2.7-positive.

The cell cycle and terminal stage of apoptosis were assessed by DNA content analysis. Floating cells were pooled with trypsinized adherent cells, washed twice with Hank's balanced salt solution and incubated for 4 h at 4ºC in 50 μg/ml propidium iodide (PI), 10 mM Tris, 0.1% TRITON X-100, 0.07% RNase and 0.1% sodium chloride, and analysed by flow cytometry using the manufacturer's analysis software. The percentage of sub-G1 gated signals reflected the proportion of apoptotic cells. For the cell cycle distribution, DNA content histograms were analyzed using the MultiCycle® for Windows (Phoenix Flow Systems, USA).

Gene expression analysis

To identify the expression patterns of p53-related genes, we used GEArray Q series Human P53 Signalling Array HS-027 macroarray membranes (Superarray Bioscience Corp., USA). H1299 and Saos-2 cells were transfected with pBluescript, wild-type p53 cDNA, and TAp73β cDNAs, individually or in combination, prior to UV exposure (40 KJ/m^2). The total RNA was isolated with the QIAGEN RNeasy system. The total RNA (4 μg) was reverse transcribed (Promega Corp., USA), and the probe labelled using the GEA labelling buffer mix (Biotin-16-dUTP; Roche Diagnostics, Switzerland). After the hybridization of the biotinylated cDNAs, the signal was generated with the streptavidin-alkaline phosphatase system. Signals were captured using a cooled CCD camera (Alpha Innotech Corp., USA).

Real-time PCR

cDNA synthesis was carried out using M-MLV reverse transcriptase (Invitrogen Corp., USA). Triplicate samples were subjected to quantitative PCR analysis using SYBR Green. The relative quantitation of gene expression was determined by the comparative C_T method. The amount of the target was normalized to *ACTB* as an endogenous reference and relative to mock tranfected cells as a calibrator.

The primer pairs used for each gene were:

DR5	forward 5'GGGAAGAAGATTCTCCTGAGATGT'3
	reverse 5'ACATTGTCCTCAGCCCCAGGTCG'3
$p21^{WAF1}$	forward 5'CTGGAGACTCTCAGGGTCGAA'3
	reverse 5'AAGATGTAGAGCGGGCCTTTG´3
bax	forward 5'AGAGGATGATTGCCGCCG'3
	reverse 5'CAACCACCCTGGTCTTGGATC'3
NoxA	forward 5'CTGTCCGAGGTGCTCCAGTT'3
	reverse 5'TCCTGAGTTGAGTAGCACAC'3
beta-actin	forward 5'GGAACGGTGAAGGTGACAGC'3
	reverse 5'ACCTCCCCTGTGTGGACTTG'3

For the evaluation of the p63 and p73 cell status, RNA was extracted using the Trizol method. cDNA was made from 1 μg of RNA using a Cloned AMV First-Strand cDNA Synthesis Kit, according to the manufacturer's instructions (Invitrogen Corp., USA). Quantitative PCRs were performed for individual p63 and p73 isoform using multiparametric kits (Search LC, Germany) analysed on a LightCycler (Roche Diagnostics, Switzerland), as previously described by Thurfjell *et al.* [17]. To evaluate the p53 expression status, we used the method described by Boldrup *et al.* [18].

Densitometry

Protein and bound biotinylated cDNA levels were evaluated by the densitometric analysis of photographic material. TotalLab software (Nonlinear Dynamics) was used to compute the relative level of protein or biotinylated

cDNA. In the macroarrays, the raw signal intensities were corrected for the background by subtracting the signal intensity of the average of the three negative controls (pUC18 cDNA) and the four blanks, and are expressed as fold changes given as the relative expression ratio of gene:housekeeping gene. Any signal whose raw intensity was less than 150% of the background was treated as a background signal, and thus not detectable.

RESULTS

The *in vitro* binding activity of TAp73 isoforms

We performed EMSA with seven p53 responsive elements (*bax*, *gadd45*, *mdm2*, $p21^{WAF1}$, *ras*, RGC and the ideal p53-binding sequence CON) in the form of double-strand oligonucleotides, and compared the *in vitro* binding activity of the four major C-terminal TAp73 isoforms – TAp73α, TAp73β, TAp73γ and TAp73δ. As a negative control, we used non-specific oligonucleotide sequences (mutant $p21^{WAF1}$), which showed no binding of p53 or any p73 isoform. The specificity of the complexes was confirmed by the antibody super-shift. To compare the binding activity of different TAp73 isoforms, we used purified Sf9-expressed p53 protein as a standard, designated as 100% densitometrical intensity.

The levels of the TAp73 isoforms synthesized by the *in vitro* transcription/translation system were characterised using the anti-HA antibody (Fig. 1A). These results confirmed the expression of all the isoforms at a comparable level and in concentrations available for the DNA-binding experiments. The specificity of interactions between the TAp73 proteins and DNA (oligonucleotide) was confirmed by the super-shift of these complexes using the anti-HA antibody (Fig. 1B)

TAp73δ exhibited the highest binding potential and TAp73γ the weakest binding potential towards all the tested p53-responsive elements (Fig. 1C). The longest isoform, TAp73α, binds weakly to all the oligonucleotides, and TAp73β generally binds to a higher degree than TAp73α. However, there is clear heterogeneity of the relative binding abilities of the different isoforms to different promoters, compared both to each other and to p53.

The transactivation potential of TAp73 isoforms

To compare the transactivation potential of the TAp73 isoforms alone and in combination with p53, we used H1299-RGC. All the TAp73 isoforms transactivate the RGC promoter in these cells. Surprisingly, the most transcriptionally active isoform was TAp73β, whereas TAp73δ shows the highest binding activity to this sequence. Surprisingly, TAp73α has a similar activity to p53, and TAp73β has an even higher activity. Co-transfection of p53 with TAp73 does not significantly affect p73 transactivation (Fig. 2).

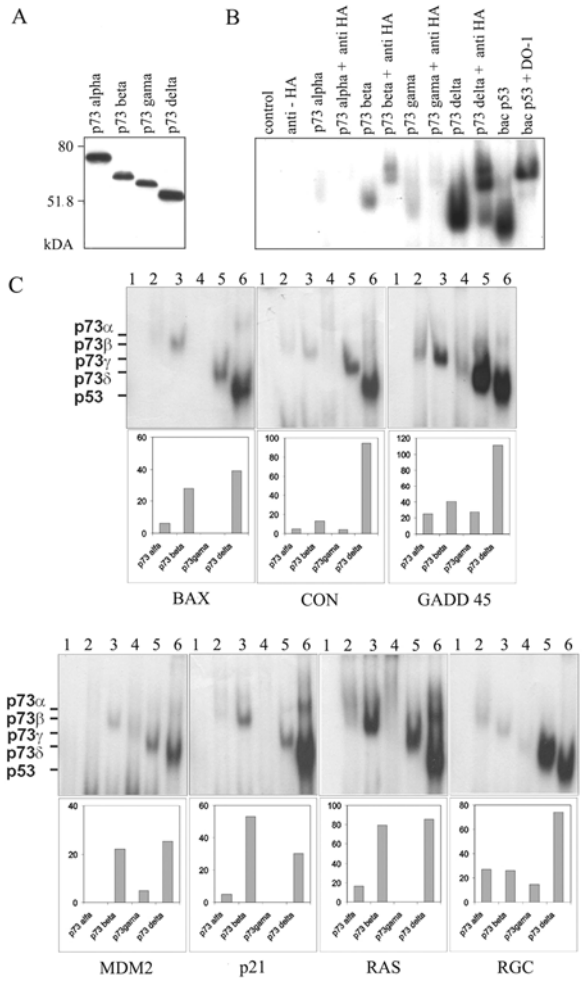

Fig. 1. Translation of the HA-tagged TAp73 isoforms and their binding to p53-responsive gene promoters. A – The Western-blot analysis of the *in vitro* transcribed/translated HA-tagged isoforms TAp73α, TAp73β, TAp73γ and TAp73δ. 1 μl of IVTT protein mixture was loaded on the gel and the anti-HA antibody was used for immunodetection. B – The EMSA analysis of the specific binding of the TAp73 isoform to $p21^{WAF1}$ oligonucleotide. The complex of the isoforms HA-TAp73α, HA-TAp73β and HA-TAp73δ with the oligonucleotide $p21^{WAF1}$ was supershifted using anti-HA antibody, proving the specificity of the TAp73-DNA interactions. The complex of the p53 protein and $p21^{WAF1}$ oligonucleotide was supershifted using DO-1 antibody. An irrelevant oligonucleotide (mutant $p21^{WAF1}$: GAAAATTTCCCAACATGTTG [19]) was used as a negative control in combination with HA-TAp73β. C – EMSA. The lanes containing bands corresponding to the complex of the oligonucleotide and a given *in vitro* translated isoform are indicated as follows: negative control (respective oligo sequence only) (lane 1), HA-TAp73α (lane 2), HA-TAp73β (lane 3), HA-TAp73γ (lane 4), HA-TAp73δ (lane 5) and the baculovirus-expressed p53 protein active for DNA binding (lane 6). The bar graphs show only the intensities of the DNA binding of the TAp73 isoforms. Band intensities were standardized against p53, which was assigned an intensity of 100%. Three parallel experiments were done with similar results, and the data from one representative experiment is shown.

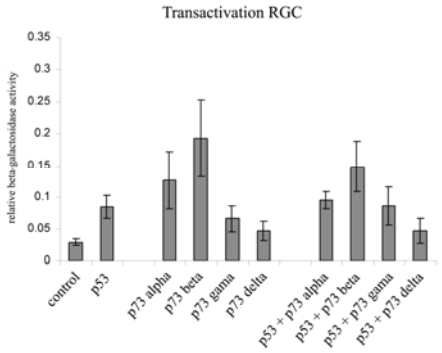

Fig. 2. The transactivation ability of TAp73 isoforms. H1299-RGC cells were transiently transfected with plasmids carrying the HA-tagged TAp73 isoforms, p53, or the control plasmid without the coding gene. β-galactosidase activities were measured 24 h post-transfection. All the experiments were run in triplicate, and the data shown is the mean enzyme activities ± SEM.

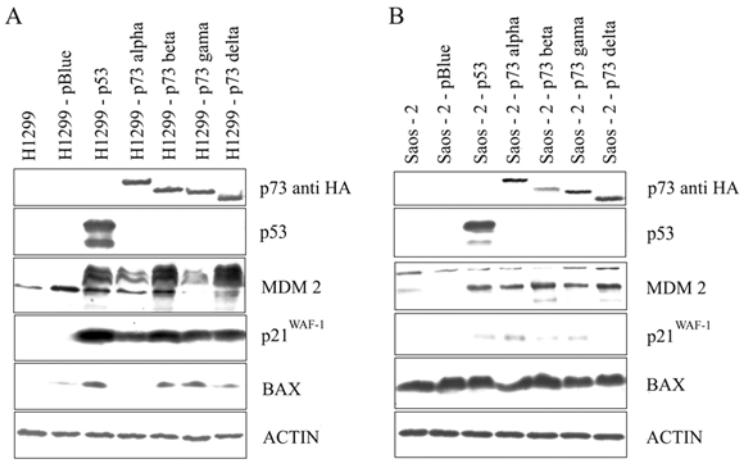

Fig. 3. The Western blot analysis of p53, TAp73, $p21^{WAF1}$, MDM2 and BAX proteins after the transient transfection of the TAp73 isoforms. A – An analysis of the protein level of TAp73 isoforms and the induction of MDM2, BAX and $p21^{WAF1}$ proteins after the transient transfection of TAp73 isoforms in the H1299 cell line. H1299 (p53-/-) cells were transfected and the proteins detected using the antibodies DO-1/HA for transfected p53/TAp73 isoforms; 2A9 for MDM2, 118 for $p21^{WAF1}$, Ab-5 for BAX and AC-40 for beta-actin (the loading control). B – An analysis of the protein level of TAp73 isoforms and the induction of MDM2, BAX and $p21^{WAF1}$ proteins after the transient transfection of TAp73 isoforms in the Saos-2 cell line. Saos-2 (p53-/-) cells were transfected, and the proteins detected using the antibodies DO-1/HA for the transfected p53/TAp73 isoforms, 2A9 for MDM2, 118 for $p21^{WAF1}$, Ab-5 for BAX and AC-40 for beta-actin (the loading control).

Next, we evaluated the level of the p53 target proteins $p21^{WAF1}$, MDM2 and BAX in H1299 and Saos-2 cells. The TAp73β and TAp73δ isoforms were the most active in the induction of MDM2 and $p21^{WAF1}$, with a similar effect to p53. TAp73α and TAp73γ activated both proteins weakly in H1299 cells compared to the other isoforms (Fig. 3A). BAX protein was induced very poorly in this cell line. Conversely in Saos-2 cells, we obtained weak induction of MDM2 and $p21^{WAF1}$ compared to the strong induction of BAX protein (Fig. 3B).

The ability of TAp73 isoforms to induce apoptosis and cell cycle arrest

We studied apoptosis induction in the p53(-/-) cell lines Saos-2 and H1299 in the early stages using Apo2.7 detection and in the late stages using DNA content detection. Using both cell lines, it is evident that individual TAp73 isoforms differ in their ability to induce apoptosis in the absence of UV treatment, and that the presence of wild-type p53 changes the levels of apoptosis induced by

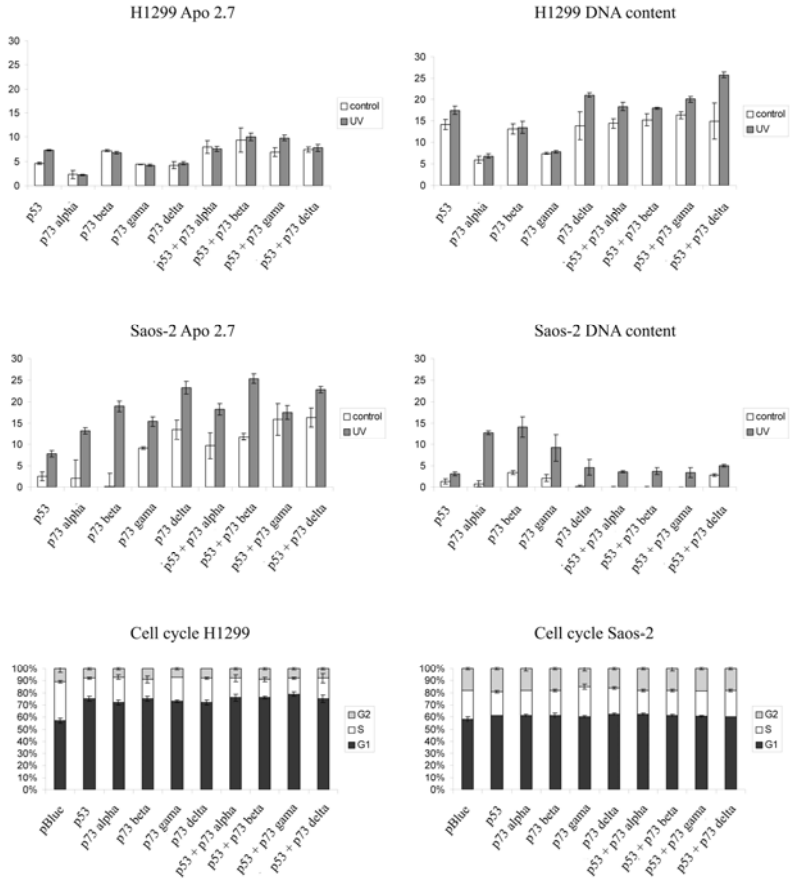

Fig. 4. The ability of TAp73 isoforms to induce apoptosis and cell cycle changes. Transfected cells were assessed for the degree of apoptosis with Apo2.7 or $subG_1$ DNA content, and assessed for the stage of the cell cycle.

TAp73. While the single transfection of H1299 cells with wild-type p53 or TAp73 isoforms induces high levels of late-stage apoptosis, transfection to Saos-2 cells does not induce detectable levels of late-stage apoptosis. Co-transfection of the p53 and TAp73 isoforms does not lead to synergistic effects in either early- or late-stage apoptosis in either cell line. After treatment with UV, early-stage apoptosis is more dramatically increased in transfected Saos-2 cells. Co-transection of p53 or TAp73 followed by UV exposure produces apoptosis levels that are similar to the values of the single transfectants in H1299 cells, whereas there is an inhibition of UV-induced apoptosis in co-transfected Saos-2 cells compared to single transfectants. On the other hand, the early stage of apoptosis in Saos-2 cells reaches the highest rates in the experiment. There are further differences between the effects of p53 and TAp73 on cell cycle regulation, where transfection of H1299 increased the percentage of cells in G1 and reduced those in the S phase, whereas transfection of Saos-2 did not influence the cell cycle distributions (Fig. 4).

The analysis of the gene expression associated with a p53-mediated signal transduction

Tab. 2 details the relative changes in the gene expression in response to transient transfection with p53, TAp73β or their combination in both Saos-2 and H1299 cells. A detailed analysis of every single gene is outside the remit of this report, and only the group of genes involved in the regulation of either the cell cycle or cell death are discussed. There is enhanced transcription of several genes that are responsible for mitochondrial apoptotic sensitization, including *ASPP2*, *APEX* and *bax* in Saos-2 cells. In H1299 cells, the over-expressed factors connected with the death receptor pathways are the Trail death receptor (*DR5*), *PIDD*, *TRAFs*, *ADO22* and *FAF1*. In addition, we found that $p21^{WAF1}$, the major regulator of p53-mediated growth arrest, is induced to a much higher extent in H1299 than Saos-2, accounting for the observed alterations to cell cycle

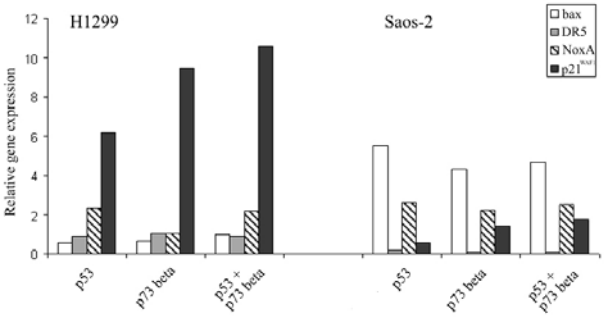

Fig. 5. The determination of the gene expression in transfected H1299 and Saos-2 cells. Real-time PCR was used to quantitate the changes in *bax, DR5, NoxA* and $p21^{WAF1}$ gene expression. The data is represented as fold-change over an empty vector control and normalised to *ACTB*.

Tab. 2. The effect of transfected wild-type p53 and TAp73β individually or together on the expression of p53-associated genes connected with cell cycle control or apoptosis in Saos-2 and H1299 cells exposed to UV 12 h previously.

Gene	Saos-2				H1299			
	Mock	wt p53	TAp73 beta	wt p53 + TAp73 beta	Mock	wt p53	TAp73 beta	wt p53 + TAp73 beta
PUMA/BBC3	1.00	1.61	1.87	1.54	1.00	1.57	1.45	0.96
NoxA	1.00	3.21	2.54	2.04	1.00	1.72	0.96	0.73
bax	1.00	5.41	5.23	2.23	1.00	0.57	1.34	0.28
cathepsin	1.00	1.36	1.55	0.66	1.00	1.73	3.95	1.71
APEX/Ref-1	1.00	1.62	2.54	1.72	1.00	1.06	1.14	0.75
ASPP2	1.00	1.39	1.99	0.77	1.00	1.18	1.73	0.71
bcl-2	1.00	0.59	1.55	0.52	1.00	7.24	27.82	29.92
Rel A	1.00	5.12	5.30	2.04	1.00	1.93	2.29	1.70
cdk1 (cdc 2)	1.00	2.35	2.36	1.13	1.00	1.39	0.74	0.42
$p21^{WAF1}$	1.00	1.67	2.64	2.47	1.00	9.77	7.09	6.20
p14 (ARF)	1.00	0.45	1.13	0.64	1.00	5.33	3.94	2.62
DR5	1.00	1.71	1.65	0.92	1.00	4.71	2.33	4.92
ADO22	1.00	0.74	0.92	0.39	1.00	1.13	1.30	0.83
PIDD	1.00	2.18	1.86	0.38	1.00	1.42	2.96	1.82
TRAF1	1.00	1.49	1.18	0.23	1.00	1.22	1.42	0.97
TRAF4	1.00	2.79	2.88	1.09	1.00	6.72	3.69	3.46
TRAF5	1.00	2.17	1.40	0.23	1.00	1.24	1.54	1.27
FADD	1.00	7.88	10.50	1.63	1.00	1.50	1.26	3.04
FAF1	1.00	1.67	4.16	0.69	1.00	2.18	8.51	2.23
PML	1.00	1.48	1.67	1.46	1.00	1.37	1.28	0.96
MAP4	1.00	0.77	0.79	0.33	1.00	1.43	2.02	1.84
beta-actin	1.00	0.96	0.94	0.90	1.00	1.02	1.05	1.02
cyclophilin A	1.00	0.95	1.03	0.98	1.00	1.02	0.98	0.98

distributions in H1299 but not Saos-2 cells. Other differences between Saos-2 and H1299 cells include over-expression of *Cdc2* (CDK1) kinase, *PML* and *MAP4* in Saos-2 compared to H1299 cells.

To validate the results obtained from the cDNA macroarrays, we analyzed the expression of selected genes via real-time PCR in both the H1299 and Saos-2 cell lines (Fig. 5). We unambiguously confirmed the strong induction of the $p21^{WAF1}$ gene in H1299 cells after the transient transfection of p53 and TAp73β, both individually and in combination; this was in contrast to the situation in Saos-2 cells. *Bax* expression was significantly induced in Saos-2 cells after transfection, by contrast to H1299 cells, which showed an identical or decreased mRNA level

in comparison to the mock transfection (Fig. 5). Furthermore, real-time PCR also validated trends in the expression of *DR5* and *NoxA* genes, revealed as significant by macroarray analysis, indicating the involvement of different signalling pathways in H1299 and Saos-2 cell lines in response to the strong expression of transiently transfected p53 or TAp73β, or p53 and TAp73β together.

DISCUSSION

An association between a wild-type status for p53 and over-expression of p73 has been observed in many studies [4, 5]. It indicates the possible oncogenic role of p73 in carcinogenesis when p53 is not altered. The *TP73* gene has two distinct promoters and codes for protein isoforms with opposite effects: while the transactivation-proficient TAp73 isoforms show p53-like pro-apoptotic effects, the amino terminal-deleted ΔTAp73 forms have anti-apoptotic functions [20]. In addition, there is extensive splicing of 3' exons that produce p73 proteins with different C-termini, suggested to influence transcriptional activity [1, 6]. To understand the function(s) of these isoforms, we analyzed how C-terminal splicing affects DNA binding to p53-consensus sites. TAp73δ exhibited the highest *in vitro* binding potential towards all the tested p53-responsive elements. The longest isoform, TAp73α, binds weakly to all oligonucleotides, in keeping with other data showing that C-terminal deletion of TAp73α activates the protein for DNA binding [21]. The TAp73γ isoform has the weakest DNA binding to all the tested oligonucleotides, although the C-terminus is shorter than TAp73β. Therefore, either the frame-shift splicing of exons 11 and 12 of *TP73* in TAp73δ results in C-terminal amino acids that inhibit the protein-DNA interaction, or the C-terminus of TAp73β contains activating sequences. However, we found that DNA binding does not directly equate with transcriptional activation of the standard p53-responsive element, RGC. In H1299-RGC cells, TAp73β has the highest transcriptional activity, inducing activation of pRGCΔfoslacZ, whereas p53 and TAp73δ show the highest DNA-binding. Thus, like our previous observations for p53 DNA binding and transcriptional activation [22], TAp73 isoforms also show discrepancies between these two properties, indicating that the identification of binding does not necessarily indicate transcriptional regulation.

By analysing the cell cycle and apoptosis triggered by TAp73 isoforms alone or in co-operation with p53, we found that the mechanisms of TAp73-induced apoptosis are different in Saos-2 and H1299 cells, although the two cell lines are p53-null. It is evident that individual TAp73 isoforms differ in their ability to induce apoptosis, and that wild-type p53 in the cell changes the level of apoptosis induced by particular TAp73 isoforms. To understand the molecular basis for these different responses, we determined the mRNA expression of key genes involved in p53 signalling in the two cell lines transiently transfected with TAp73β as the most apoptotic active isoform. We found that Saos-2 cells showed high levels of apoptosis induction at the mitochondrial level (Apo2.7),

and that this is associated with the induction of mitochondrial-related apoptotic genes. Neither Apo2.7-detected apoptosis nor the induction of these mitochondrial-related apoptotic genes are seen in H1299 cells. By contrast, H1299 cells show high levels of late-stage apoptosis, and this is associated with a higher expression of death receptor pathway genes. We also found that TAp73 influences the cell cycle distribution in H1299 cells that induce $p21^{WAF1}$ mRNA, but that the cell cycle is not affected by TAp73 in Saos-2 cells, since these do not induce $p21^{WAF1}$.

The determination of gene expression in Saos-2 cells showed an enhanced transcription of several genes that can be responsible for mitochondrial apoptotic sensitisation, such as *ASPP2*, *APEX* and *bax*. On the other hand, as a result of inactive Rb, Saos-2 cells overexpress Cdc2 (CDK1) kinase, which prolongs S phase entry, while over-expressed factor PML stabilises active p73, and the further over-expressed kinase MAP4 [23], which is inhibited by p53 [24], can delay irreversible apoptosis changes in the case of the parallel presence of p73 isoforms and wild-type p53.

H1299 cells only show a low level of apoptotic sensitisation with a slight additive effect in the case of the presence of TAp73 isoforms together with wild-type p53 protein. Furthermore, unlike Saos-2 cells, the macroarray results indicate a raised expression of factors connected with the death receptors. These observations concur with a recently described mechanism of p73-dependent Fas-mediated apoptosis showing that p73 transcriptional activity sensitized cells to apoptosis through death receptors in a caspase-dependent pathway [25, 26]. Similarly, we also observed the up-regulation of genes involved in the activation of the TRAIL receptor. If we consider gene expression data together with the fact that mitochondrial apoptotic sensitization was low, we can speculate about a dominant role of TRAIL-mediated apoptosis in response to a wide range of different apoptotic stimuli in cancer cells like H1299. Moreover, elevated expression of Rel A, a direct regulator of mitochondrial active anti-apoptotic factor Bcl-xl, and the increased level of Bcl-2 can also contribute to the protection of the mitochondria against sensitization to apoptosis in H1299 cells [27].

Thus, our data clearly indicates that the same TAp73 isoform has dramatically different effects on apoptosis and cell cycle arrest, and demonstrates that the cellular background, independently of p53, determines the apoptotic and growth arrest pathways that are transcriptionally activated by TAp73β.

The different expression profiles of genes affecting the cell cycle and death induced by p53, TAp73β, or their combination indicates that in different cells these proteins have distinct functional capabilities and modes of action. Recent data indicated that p73 expression is an important contributory factor to the therapeutic response in a variety of common human cancers in cooperation with p53. Our data suggests that different types of tumours exist in terms of the p73 response following chemotherapy, where some tumours will exhibit p73-mediated growth arrest and death-receptor mediated apoptosis (H1299-like), whereas others will exhibit more robust apoptotic responses due to the activation

of mitochondrial factors (Saos-2-like). Given that cytotoxic drugs and radiotherapy, when successful, induce apoptosis, and that their effects rely predominantly on the activation of mitochondrial pathways [28], it can be expected that the activation of mitochondrial factors will provide better therapeutic success for patients. It is important to study the effects of p73-mediated responses more fully to delineate and understand the potential for tumour-specific responses and their impact on patient survival. Understanding these differences may help to direct the most appropriate use of therapy and may eventually lead to new therapeutic opportunities for a variety of human tumours based on the differential activation of target genes by p73 isoforms.

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