Adenovirus-mediated TA-p73β gene transfer increases chemosensitivity of human malignant melanomas

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Malignant melanoma is the most aggressive form of skin cancer and has proven to be highly resistant to conventional chemotherapy. Intriguingly, the p53 tumor suppressor, a main mediator of chemoresistance in other tumor types, is rarely mutated in melanoma. However, we have previously shown that anti-apoptotic isoforms of p73 (ΔTA-p73), another member of the p53 family, are overexpressed in metastatic melanomas. ΔTA-p73 can oppose the pro-apoptotic functions of p53 and full length p73, and thus it could contribute to melanoma chemoresistance. In this study, we use an efficient adenoviral-based gene transfer approach to introduce a transcriptionally active form of p73 (TA-p73β) in melanoma cells, with the objective of overcoming drug resistance. Interestingly, TA-p73β significantly sensitized 5 out of 7 aggressive melanoma cell lines to the standard therapeutic agents adriamycin and cisplatin. More importantly, TA-p73β displayed a synergistic effect *in vivo* allowing adriamycin or cisplatin to block melanoma cell growth in mouse xenograft models ($p < 0.05$). In summary, our data show that Ad-mediated TA-p73β gene expression can markedly sensitize a subset of melanoma cell lines to adriamycin and cisplatin *in vitro* and *in vivo*, suggesting a new chemosensitization strategy for malignant melanomas.

Keywords: apoptosis; chemotherapy; gene therapy; melanoma; p73.

Introduction

Malignant melanoma has been one of the fastest rising malignancies in the last decades, with a life time risk currently of 1/53. This highly aggressive form of skin cancer is curable when resected at an early stage. In its metastatic stage, melanoma has an extremely poor prognosis since chemotherapy is ineffective due in part to the intrinsic and/or extrinsic resistance of melanoma cells to systemic treatment with anti-neoplastic agents.[1] Most anticancer drugs kill susceptible cells through the induction of apoptosis. Therefore alterations of apoptotic pathways leading to apoptosis defi-

ciency represent a potent mechanism conferring drug resistance on melanoma cells.[2] Acquired apoptotic defects during melanoma progression involve activation of anti-apoptotic factors, e.g. survivin,[3] inactivation of proapoptotic factors, e.g. Apaf-1,[4] and upregulation of survival signals, e.g. B-Raf.[5] Restoration of the apoptotic pathway, e.g. by suppression of survivin[3] or activation of Apaf-1,[4] has been shown to increase the sensitivity of melanoma cells to chemotherapeutic agents. As an important molecular component of apoptosis p53 influences the resistance to chemotherapy in malignant tumors. However, melanomas show a low frequency of p53 mutations despite their extreme chemoresistance.[6] One explanation of this paradox is that disruption of pathways upstream and/or downstream of p53 could functionally replace p53 loss during melanomagenesis, e.g. inactivation of p14ARF[7] or Apaf-1.[4]

The p53 family member p73 shares significant sequence homology with p53. However, in contrast to p53, p73 gives rise to multiple protein isoforms due to alternative promoter utilization and alternative mRNA splicing.[8] The TA isoforms of p73, e.g. TA-p73α and TA-p73β, contain the N-terminal transactivation domain. These TA-73 isoforms can transactivate p53 target genes and induce apoptosis. In addition, TA-p73 is induced by a wide variety of chemotherapeutic drugs, such as adriamycin and cisplatin[9–12] and blocking of TA-p73 function has been shown to enhance chemoresistance.[11,13] Other p73 isoforms, namely ΔTA-73, lack the transcriptional activation domain and block the pro-apoptotic function of p53 and TA-p73.[14,15] Thus, overexpression of ΔTA-73 isoforms can block chemotherapy induced apoptosis in tumor cells that retain wild-type p53.[15,16]

Interestingly, a strong expression of p73 was found in metastatic melanomas, suggesting that p73 might function as a positive regulator of tumor growth in the progression from primary melanoma to metastases.[17] Detailed analyses of p73 isoforms revealed an overexpression of ΔTA-73 isoforms in metastatic melanomas, suggesting a potential function of ΔTA-p73 in melanoma progression due to inhibition of p53 and TA-p73 function.[18]

These findings indicate that the ratio of TA-p73 to ΔTA-p73 may be an important determinant of drug response.

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Thus, we hypothesized that restoring TA-p73 function by overexpression could confer sensitivity to chemotherapy-induced apoptosis in tumor cells. Here we demonstrate that upregulation of TA-p73β expression by adenoviral gene transfer enhances chemosensitivity to adriamycin and cis-platin in a subset of melanoma cell lines, both *in vitro* and *in vivo.* Our data emphasizes the need of a comprehensive analysis of p53 homologues in melanoma cells to understand the molecular basis of drug resistance in this aggressive neoplasm.

Materials and methods

Cell lines and culture conditions

Human melanoma cell lines Mel1 (SK-Mel-19), Mel2 (SK-Mel-29), Mel3 (SK-Mel-103), Mel4 (SK-Mel-147), Mel5 (G-361), Mel6 (UACC-257), all wild-type p53, were derived from metastatic melanomas from patients. Mel1-Mel6 were cultured in DMEM. DMEM was supplemented with 10% heat-inactivated fetal bovine serum (PAA Laboratories, Coelbe, Germany) and 100 units/ml penicillin/streptomycin (GIBCO). Mel7 (WM115; generously provided by Dr. Birgit Schittek; Department of Dermatology, Eberhard-Karls University, Tübingen, Germany), wild-type p53, derived from primary melanoma, was cultured in MCDB. MCDB was supplemented with 20% Leibowitz cell culture medium (L-15; GIBCO), 2.5% heat-inactivated FBS, 100 mg/ml gentamycin (PAA), and 40 U/ml insulin (Aventis). Cells were cultured in a 5% CO_2 incubator at 37°C. Medium was changed every 3 days.

Adenoviral vectors

Three replication-deficient recombinant adenoviral vectors, Ad-GFP, Ad-GFP-TA-p73β, and Ad-TA-p73β, expressing transgenes under the control of the CMV-promoter have been described previously.[19,20] All vectors were propagated in 293 cells, purified by CsCl buoyant density centrifugation and titrated by standard plaque assays.

Chemotherapeutic agents

For *in vitro* use adriamycin (ADR, doxorubicin; Pfizer) and -cisplatin (cDDP, [cis-platinum(II)-diammine dichloride]; GRY) were diluted in culture medium before addition to cell cultures. For *in vivo* use drugs were diluted in 100 μl 0.9% saline immediately before i.p. administration into nude mice.

Adenovirus infection and drug treatment

1×10^6 melanoma cells were plated in 10-cm tissue culture plates. After 24 h, cells were infected with adenoviral vector at a multiplicity of infection (MOI 10 to 100) which allows 100% transduction of each cell line. For combination treatment, chemotherapeutic agents were added to infected cells at a concentration of 0.3 μg/ml for adriamycin and 3 μg/ml for cisplatin. Cells were harvested 3 days after infection.

Real-time RT-PCR

Total RNA was isolated with the RNeasy Mini Kit (Qiagen) according to the manfactures protocol and 1 μg RNA was used for reverse transcription with Omniscript RT (Qiagen) using random hexamers (Applied Biosystems). PCR reactions contained 65 ng cDNA, 1 × Lithos qPCR^{TM} SYBR Green I Master Mix (Eurogentec), 150 nM of each primer, 3.5 mM $MgCl_2$ and 0.5 mg/ml BSA (Roche) and were carried out in a LightCycler (RocheDiagnostics). Amplification products for TA-p73, p73Δex2, p73Δex2/3, $\Delta N'$-p73, ΔN-p73, p21$^{WAF1/CIP1}$ and S9 ribosomal protein (S9ribP) were verified by melting curves, agarose gel electrophoresis and direct sequencing. PCR conditions, primer sequences and control plasmids were described previously.[14,18]

Western blotting

Cells were lysed in RIPA buffer (50 mM Tris-Cl, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS) and total protein concentration was quantitated by Bradford assay. Equal amounts of each sample were separated by SDS-PAGE, transferred to nitrocellulose membranes (Amersham-Biosciences; Freiburg, Germany) and probed with antibodies against cleaved Caspase-3 (#9661), full-length PARP (#9542), or cleaved PARP (#9541) from Cell Signaling Technology (Frankfurt, Germany). Reprobing of the blot with actin antibody (Santa Cruz Biotechnology; #sc-1616, Heidelberg, Germany) served as gel loading control. Primary antibodies were detected with appropriate secondary antibody-horseradish peroxidase conjugates (Amersham Pharmacia, Braunschweig, Germany) according to the enhanced chemiluminescence protocol (Pierce).

Flow cytometry analysis

For flow cytometry analysis, adherent and nonadherent cells were harvested, fixed in 70% (v/v) ethanol and stained for DNA content with propidium iodide. FACS analysis was performed at 3 days after infection. The data from 10,000 cells were collected and analyzed using CellQuest^{TM} soft-

ware (Becton Dickinson). The subdiploid population was calculated as an estimate of the apoptotic cell population. Analysis was carried out in a FACSCalibur flow cytometer (Becton Dickinson, San Diego, USA) using CellQuest Software.

Hoechst 33342 staining

At 3 days post infection Hoechst 33342 was added to the cell culture medium at 1 μg/ml. Cells were incubated at $37°C/5\%$ CO_2 and subsequently subjected to fluorescence microscopy.

TUNEL assay

The immunohistochemic detection of apoptosis in formalin-fixed, paraffin-embedded tumor tissues was performed by using the Promega DeadEnd^{TM} Fluorometric TUNEL System. The TUNEL reaction mixture (50 μl) was applied to deparaffinized and rehydrated tumor sections and the slides were incubated for 60 min at $37°C$. The tissues were counterstained with propidiumiodide and apoptotic cells were determined by microscopic examination. For each tumor three different slides were examined.

Animal experiments

Tumors were established by subcutaneous injection of 5×10^6 Mel3 or Mel4 cells into the right flank of athymic BALB/c nu/nu mice (6–8 weeks of age). Mice with visible and palpable tumors received three direct intratumoral injection of purified Ad-TA-p73β (5×10^9 pfu) or Ad-GFP (5×10^9 pfu) every 3 days. Virus was diluted in total volume of 100 μl of 0.9% saline and administered in a single pass of a 27-gauge hypodermic needle using constant infusion pressure. Beginning on day 7, mice were treated with ADR (1 mg/kg), cDDP (6 mg/kg) or 0.9% saline via i.p. injections every 3 days, for a total of five treatments. Each experimental group consists of 6 mice. The individual groups received the following treatment combinations: group1, Ad-GFP+0.9% NaCl; group2, Ad-GFP+ADR; group3, Ad-GFP+cDDP; group4, Ad-TA-p73β+0.9% NaCl; group5, Ad-TA-p73β+ADR; group6, Ad-TA-p73β+cDDP. Tumors were measured every 3 days in two perpendicular diameters using calipers, and tumor size was calculated using the formula: length (mm) × width (mm^2)/2. Animals were sacrificed when the longest diameter was greater than 15 mm or when any two measurements were greater than 10 mm. Animal experiments were performed in accordance with institutional guidelines set by the University Institutional Animal Care and Use Committee.

Statistical analysis

Statistic analysis was performed using paired two-sided Student's t test. $P < 0.05$ was considered as statistically significant.

Results

p73 expression status in melanoma cell lines

In order to determine which p73 isoforms are expressed in the melanoma cell lines, total cellular RNA was purified from cultured melanoma cells and transcript levels of TA-p73 and ΔTA-p73 (p73Δex2, p73Δ2/3, ΔN'-p73, ΔN-p73; Figure 1A) were quantified using real-time RT PCR. As shown in Figure 1B, TA-p73 expression was observed in all melanoma cell lines with highest transcript levels in Mel7 and lowest transcript levels in Mel3. ΔTA-p73 isoforms p73Δex2, p73Δ2/3 and ΔN-p73 were expressed in all melanoma cell lines, except in Mel3 (p73Δex2 and ΔN-p73 mRNA not detectable). In contrast, ΔN'-p73 transcript expression was only detectable in 3/7 melanoma cell lines (Mel2, Mel6, Mel7). Thus, all melanoma cell lines significantly expressed both proapoptotic TA-p73 and antiapoptotic ΔTA-p73 transcripts. An important issue to consider is the ratio of the two antagonistic p73 protein classes. 5/7 melanoma cell lines showed an overweight of ΔTA-p73 transcripts (ΔTA-p73/TA-p73 ratios: Mel5, 6.9; Mel1, 5.4; Mel4, 4.2; Mel7, 2.3; Mel2, 2.1), whereas 2/7 melanoma cell lines revealed an overexpression of TA-p73 (ΔTA-p73/TA-p73 ratios: Mel3, 0.8; Mel 6, 0.4). On the protein level the ΔTA-p73/TA-p73 ratio might be higher, because the ΔTA-p73 proteins were reported to be more stable.[19] However, at present, it is not known how much ΔTA-p73 activity is required to outweigh the tumor suppressor function of TA-p73. Taken together these data support a possible oncogenic function of ΔTA-p73 in the investigated melanoma cell lines.

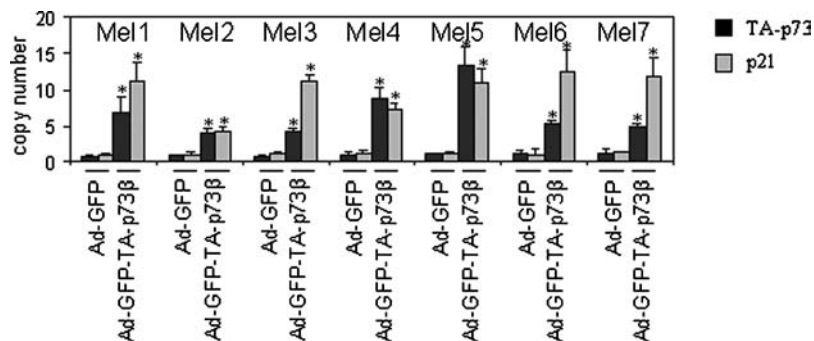## Induction of p21$^{WAF1/Cip1}$ by TA-p73β in melanoma cell lines

First, functional activity of the Ad-GFP-TA-p73β vector was tested in melanoma cell lines by measuring expression of p73 and its downstream target p21$^{WAF1/Cip1}$. Cells were infected either with Ad-GFP-TA-p73β or Ad-GFP, and transcripts levels of TA-p73 and p21$^{WAF1/Cip1}$ were quantified at 36 h after infection using real-time RT PCR (Figure 2). As suggested, Ad-GFP-TA-p73β infected melanoma cell lines showed increased TA-p73 expression in comparison to untreated controls with a fold induction ranging from 3.9 (Mel2) to 13.3 (Mel5), whereas upregulation of TA-p73 was not observed in Ad-GFP treated melanoma cells. In all melanoma cell lines, p21$^{WAF1/Cip1}$, known to be a p73-dependent gene, was significantly transactivated following

Figure 1. Expression of **N**-terminally truncated *TP73* transcript isoforms in melanoma cell lines. (A) Schematic diagram of the various ∆TA-p73 isoforms. Exon structure of individual mRNAs, the promoter, from which they are transcribed, the encoded protein and their transactivation function are as indicated. *Red*, transactivation domain; *orange*, exon 3B-derived coding sequence; *blue*, DNA-binding domain; *grey*, unique 5' untranslated region of the ∆N-p73 transcript. Primer localization is marked by arrows. (B) *TP73* transcripts (TA-p73, p73∆ex2, p73∆2/3, ∆N'-p73, ∆N-p73) were quantified by real-time PCR. The bars show the absolute copy number normalized to S9 ribosomal protein (S9ribP) transcript levels as a housekeeping gene. Errors bars indicate one SD. *n.s.* transcript expression not significant (*P*-values <0.05 were regarded as statistically significant; paired, two-sided *t*-test).
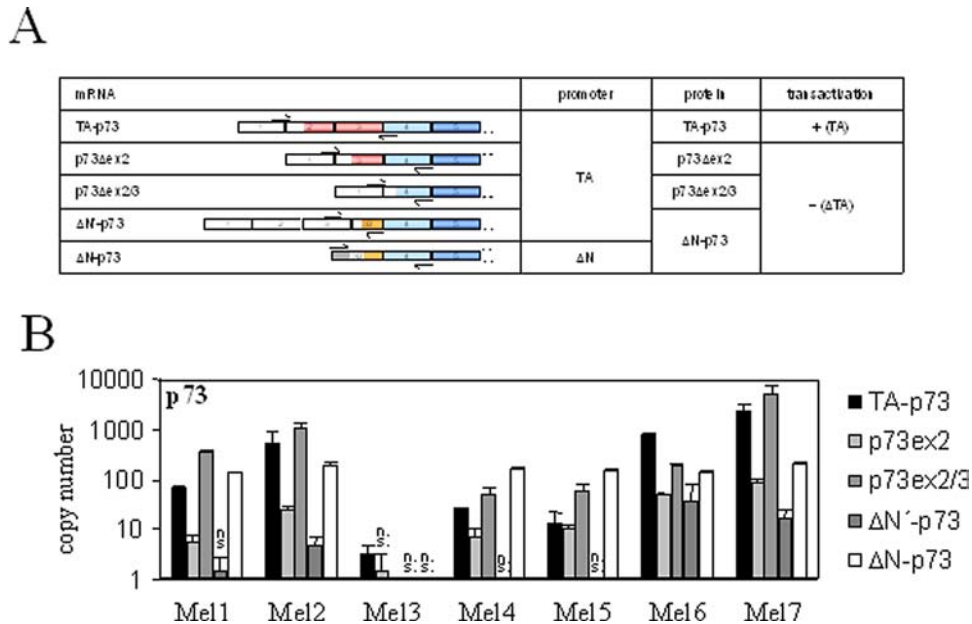
Figure 2. Expression of TA-p73 (*black bars*) and p21 (*gray bars*) transcripts in melanoma cells 36 h after infection with Ad-GFP-TA-p73β or Ad-GFP. Transcript copy numbers are calculated as fold inductions to untreated controls. Error bars indicate one SD of two independent experiments. *, Significant differences (*P* < 0.05; paired, two sided *t*-test) between Ad-GFP and Ad-GFP-TA-p73β infected cells.


Ad-GFP-TA-p73β infection resulting in a 4.1- (Mel2) to 12.6-fold (Mel6) induction. In contrast, Ad-GFP infection did not contribute to higher p21$^{WAF1/Cip1}$ transcript levels.

Induction of apoptosis by TA-p73β in melanoma cell lines

In order to examine whether apoptosis was induced by the TA-p73β protein, melanoma cell lines were infected either with Ad-GFP-TA-p73β or control virus (Figure 3). The fate of infected GFP-positive melanoma cells was followed by fluorescence microscopy. Unlike Ad-GFP infected

cells which had a normal morphology (Figure 3A), cell overexpressing TA-p73β rounded up and showed typical features of apoptotic cells characterized by membrane blebbing, cell shrinkage, and intense staining of condensed chromatin as analyzed by Hoechst 33342 staining (Figure 3B). As demonstrated in Figure 3C, increased expression of TA-p73β in melanoma cells (shown for Mel4) was accompanied by caspase-3 (Casp-3) activation and by cleavage of the 116 kD intact form of PARP to the 89 kD fragment which is intimately linked to the induction of apoptosis. Both Casp-3 and PARP cleavage became evident as early as 72 h after infection. Flow cytometry analysis of cells infected with Ad-GFP-TA-p73β revealed a sub-G1 population indicative

Figure 3. Induction of apoptosis in melanoma cells by adenoviral TA-p73β transfer. Mel4 is shown as a representative cell line. (A) Cells were stained with Hoechst 33342 at 72 h after infection and analyzed by laser scan microscopy. Infected cells are indicated as GFP positive. (B) Higher magnification (x 20) of Ad-GFP-TA-p73ß infected cells showing typical features of apoptotic cells with membrane blebbing, cell shrinkage, and nuclear condensation. (C) Caspase-3 activation and PARP cleavage analyzed by Western blotting using antibodies detecting the 19 and 17 kD subunits of cleaved caspase-3 (Casp-3), full-length PARP and the 89 kD proteolytic cleavage product. Actin was used as a loading control.
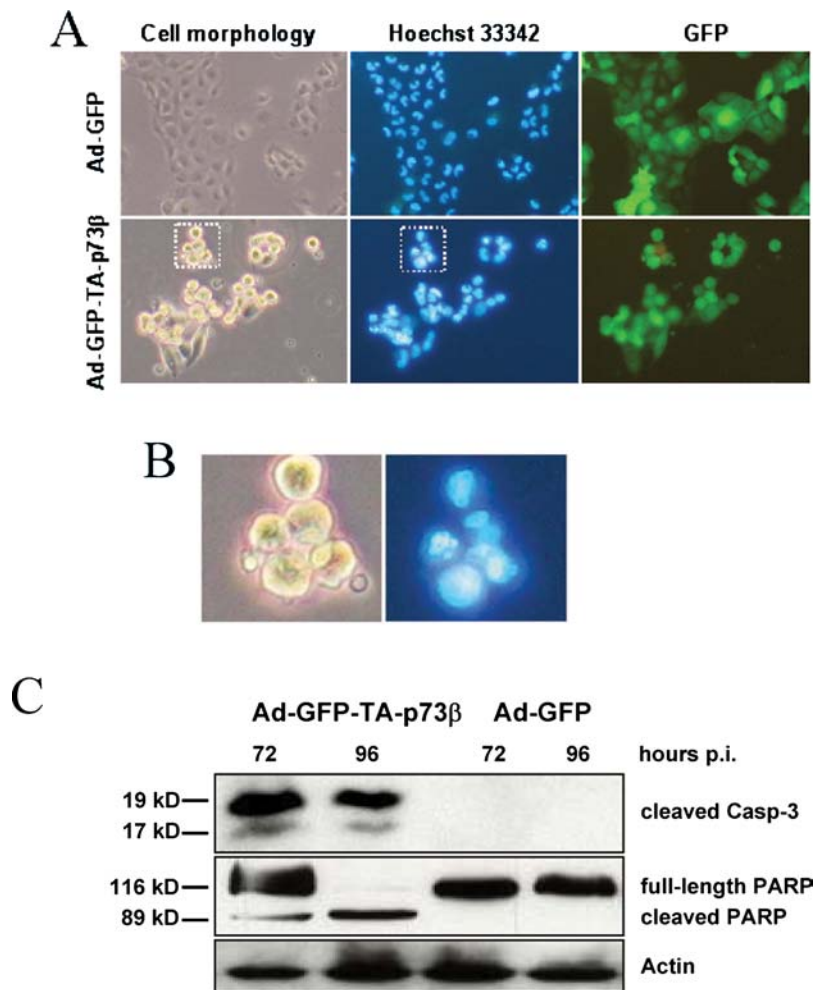
for apoptosis ranging from 7.7% (Mel6), 17.4% (Mel7), 20.6% (Mel4), 32.5% (Mel1), 36.6% (Mel2), 36.5% (Mel3) to 42.1% (Mel5) at 3 days after infection (Figure 4), indicating that the capability of TA-p73β to induce apoptosis strongly differs between individual melanoma cell lines.

Sensitization of melanoma cell lines to adriamycin and cisplatin by Ad-mediated TA-p73β gene transfer *in vitro*

Recent reports showed that endogenous TA-p73 cooperates with DNA-damaging agents to induce apoptosis in tumor cells.[9–12] Next, we investigated whether increased levels of exogenous TA-p73β in melanoma cells could enhance their sensitivity to chemotherapeutic drugs. Melanoma cells infected with adenovirus vector (Ad-GFP-TA-p73β

or Ad-GFP) were subsequently treated with adriamycin or cisplatin and harvested for cell cycle analysis. Ad-GFP-TA-p73β infection resulted in a marked sensitization of several melanoma cell lines to low concentrations of adriamycin (*ADR*) and cisplatin (*cDDP*) as measured by flow cytometry (see Figure 4A for an example of Mel4 and Figure 4B for quantification analyses with all the cell lines in this study).

In lines 6 and 7, the effect of TA-p73β showed an additive effect with ADR or cDDP (Figure 4B). In Lines 1, 2 and 5, the impact of TA-p73β on drug response was synergistic with both drugs, and in lines 3 and 4 with ADR. For example, compare the 12% or 18% killing by ADR or cDDP single agents, respectively with a 80.6% in the presence of overexpressed TA-p73β (-note that killing with TA-p73β is limited to 32.5%; see Figure 4B). In contrast, Ad-GFP infection had no obvious effect on drug sensitivity. Taken together, these data indicate that adriamycin and/or

Figure 4. Apoptotic effect of Ad-GFP-TA-p73ß and adriamycin or cisplatin combination treatment on human melanoma cells. Melanoma cells were treated with Ad-GFP, Ad-GFP-TA-p73ß, adriamycin (*ADR*), cisplatin (*cDDP*) or combinations thereof as shown. Chemotherapeutic agents were used at a concentration of 0.3 μg/ml for ADR and 3 μg/ml for cDDP. (A) Flow cytometry profiles showing DNA content (x-axis) against cell number (y-axis). Mel4 is shown as a representative sample. The percentage of cells with a sub-G1 DNA content is as indicated. (B) Flow cytometry results of two independent experiments $\pm$ SD (control *light gray*, Ad-GFP infected *dark gray*, Ad-GFP-TA-p73β infected *black*). * Significant differences ($P < 0.05$ paired, two sided *t* test) between Ad-GFP-TA-p73β and Ad-GFP infected cells or control cells.
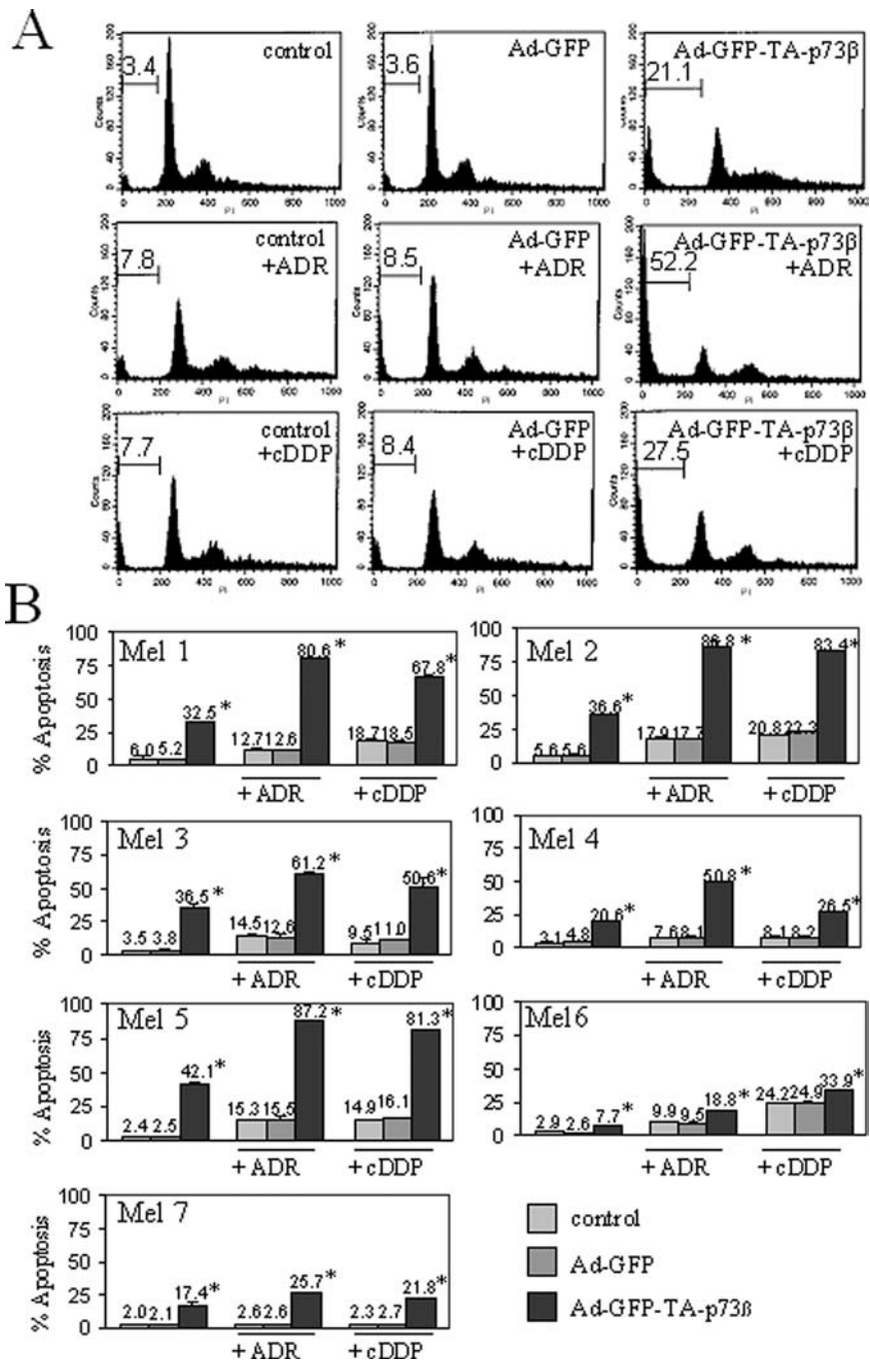

cisplatin mediated cell killing can be efficiently enhanced by TA-p73β gene expression in the majority of melanoma cell lines. Apoptosis induction in response to Ad-GFP-TA-p73β+ADR and Ad-GFP-TA-p73β+cDDP combination therapies was also confirmed by Hoechst 33342 staining (data not shown).

Antitumor effect of TA-p73β and ADR/cDDP combination therapy *in vivo*

To determine the physiological impact of the results we obtained in tissue culture systems, we analyzed the therapeutic efficacy of combination therapy on growing tumors

Figure 5 (A) *In vivo* antitumor effect of TA-p73β/ADR or TA-p73β/cDDP combination therapy. After s.c. administration of 5×10^6 Mel3 or Mel4 cells, palpable tumors were randomized to receive a local injection of Ad-GFP or Ad-TA-p73β on days 6, 9 and 12 followed by i.p. injection of ADR (1 mg/kg) or cDDP (6 mg/kg) on days 7, 10, 13, 16, 19. The total virus dose was $3 \times 5 \times 10^9$ pfu. Mice were monitored for tumor regression over 21 days. The results are expressed as the mean from each treatment group $\pm$ SD. The differences in tumor size in animals treated with Ad-TA-p73β+ADR or Ad-TA-p73β+cDDP were statistically significant compared with mice treated with Ad-TA-p73β alone, Ad-GFP alone, Ad-GFP+ ADR or Ad-GFP+cDDP (day21, $P < 0.05$; paired two-sided *t*-test). The differences in tumor size in animals treated with Ad-TA-p73β alone were statistically significant compared with mice treated with Ad-GFP alone, Ad-GFP+ADR or Ad-GFP+cDDP (day21, $P < 0.05$; paired, two-sided *t*-test). (B) TUNEL staining for apoptotic cells in melanoma tissues (representative Mel3 tumors are shown) at 72 h after intratumoral injection of 5×10^9 pfu Ad-GFP or Ad-TA-p73β. Increased apoptotis (green cells) was detected in Ad-TA-p73β treated tumor tissue. A higher magnification (x 20) of apoptotic cells is shown in the right panel.
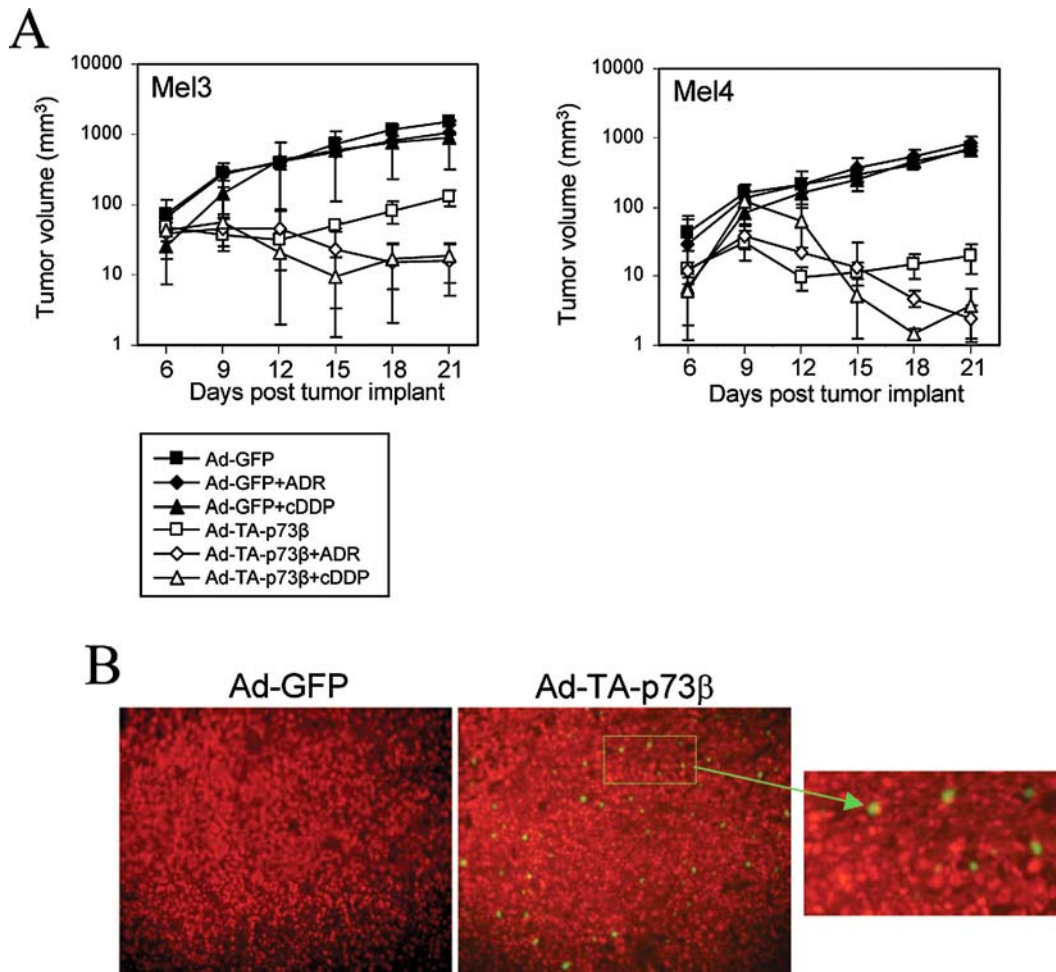
in mice. Established subcutaneous Mel3 and Mel4 tumors were treated by three intratumoral injections of Ad-GFP or Ad-TA-73β followed by five i.p. injections of either ADR or cDDP over a period of 21 days and monitored for regression (Figure 5A). Both Ad-GFP alone and in combination with either drug showed a similar effect resulting in a pronounced tumor growth. Compared to the controls, tumor growth was markedly delayed after injection of Ad-TA-p73β (day 21, $P < 0.05$; paired two-sided *t*-test). In both tumor models, a significant reduction of tumor size was observed only after combination treatment of Ad-TA-p73β and adriamycin or cisplatin (day 21, $P < 0.05$; paired two-sided *t*-test; Figure 5), which resulted in a three week longer survival of mice (data not shown). Importantly, no signs of systemic toxicity were observed in animals receiving the combina-

tion therapy. These findings are in accordance to our *in vitro* data, which demonstrated a synergistic effect of combined Ad-TA-p73β/ADR treatment and an additive effect of Ad-TA-p73β/cDDP combination therapy on cell death in Mel3 and Mel4 cells (Figure 4). Compared to Ad-GFP treated controls, a positive TUNEL staining was detected only in melanoma tissue injected with Ad-TA-p73β (Figure 5B), indicating that apoptosis induction is the mechanism of p73-mediated cytotoxicity *in vivo*.

Discussion

The success of systemic therapy for metastatic melanomas has been minimal. Chemotherapeutic drugs could theoretically target metastatic melanomas, but current treatments

do not provide a significant therapeutic benefit.[1] One of the major mechanisms that contribute to chemotherapy mediated tumor cell death is the induction of apoptosis. The extreme resistance of malignant melanoma to chemotherapeutic drugs, and the fact that apoptotic indices are typically low in melanoma tumors, particularly at advanced stages,[21] prompted an intense search for acquired apoptotic defects during melanoma progression.[2]

p53 plays a central role in the apoptotic response to DNA damaging agents. Consistent with its role in chemotherapy induced apoptosis, p53 mutations have been linked to chemoresistance in many types of cancer. Nonetheless, melanomas display a low frequency of p53 mutations.[6] One explanation for the extreme chemoresistance in the presence of wild-type p53 in melanomas is that upstream p53 regulators or downstream p53 mediators may functionally replace p53 loss during melanomagenesis. For example Apaf-1, which is an effective downstream mediator of p53-induced apoptosis, is frequently downregulated in metastatic cell lines and tumor specimens[22–24] and restoration of Apaf-1 function through gene transfer has been shown to enhance chemosensitivity.[4] However, other mechanisms should contribute to the malignant phenotype of melanoam cells since Apaf-1 overexpression does not completely restore drug response.[25] We had previously shown that metastatic melanoma cells have a dysregulated expression of different isoforms of p73, a factor that shares tumor suppressor functions with p53. Here we show that melanoma cells express a higher ratio of anti- to pro-apoptotic isoforms of p73. Reverting this ratio by overexpression of full-length p73 significantly sensitized melanoma cells to the the chemotherapeutic drugs adriamycin and cisplatin (otherwise poorly effective in melanoma).

It has been puzzling that p73, to the contrary to p53, can be upregulated in human tumors. In some cases, overexpression of p73 could even be correlated with an advanced tumor stage or poor prognostic parameters.[26,27] Moreover, various chemotherapeutic drugs, such as adriamycin and cisplatin, mediate apoptosis in cancer cells in part by upregulating p73 transcripts and stabilizating p73 protein via c-Abl.[9–12] However, the fact that anti-apoptotic isoforms can also be upregulated in tumor cells,[8,14,15] emphasizes the complexity of p73 function. The ΔTA-p73 proteins can be generated by the following two mechanisms: (a) abberant splicing (p73Δex2, p73Δex2/3, ΔN′-p73) and (b) alternative usage of a second intrinsic promoter (ΔN-p73).[8] ΔTA-p73 proteins were shown to compromise p53- and TA-p73-dependent apoptosis[16,19] and thereby to promote malignant transformation.[15,28]

Malignant melanomas have also been shown to express high levels of wild-type p73,[17,29] but also ΔTA-p73 levels (mostly TA-promoter derived p73Δex2/3 and p73Δex2 isoforms).[18] Thus, ΔTA-p73 upregulation is another possible mechanism that may functionally replace p53 loss during melanomagenesis. Here we further extend this previous data, showing that transactivation-deficient,

anti-apoptotic ΔTA-p73 isoforms are expressed in 7/7 of our melanoma cell lines, suggesting a possible role of ΔTA-p73 in apoptosis-deficiency and chemoresistance by inhibition of p53 and TA-p73 function.

Thus, we hypothesized that a therapeutic approach for sensitizing melanoma cells to chemotherapeutic drugs could be antagonizing ΔTA-p73 function and restoring p73-mediated apoptosis by overexpression of TA-p73. In melanoma cell lines both p53 and TA-p73β gene transfer has been reported to promote apoptosis.[30,31] Here we show that TA-p73β is more effective as a chemosensitizing factor. When TA-p73β was combined with adriamycin we observed a synergistic effect on apoptotic cell death in 5/7 melanoma cell lines *in vitro*. As a potential reason of why these five cell lines, and not the other two, are sensitive to TA-p73β one could expect differences in the ratio of ΔTA:TA isoforms. However, there was no significant correlation between the lack of TA-p73β-mediated chemosensitivity and the ratio of anti-apoptotic versus apoptotic p73 in these cell lines. We can thus speculate that other factors such as a variable induction of p73 downstream target genes or the failure of some cells to express the transduced gene even after 100% infection explain the difference in therapeutic outcome. Both, adriamycin and cisplatin also cooperated with TA-p73β to enhance antitumor activity in xenograft tumors using Mel3 and Mel4 cells. Most importantly, the significant drug-sensitizing effect of TA-p73β found *in vivo*, supports the development of novel therapeutic strategies aiming to block ΔTA-p73 or to exploit the TA-p73β levels that melanoma cells intrinsically express.

Acknowledgments

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