p53 Family Members Regulate the Expression of the Apolipoprotein D Gene*

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The p53 family consists of three members, p53, p73, and p63. These proteins share a high degree of amino acid sequence similarity and major functional domains. The p53 gene, the first member of the family to be identified, is the most frequent target p73, we demonstrated that silencing endogenous p73 abolishes induction of apoD transcription following cisplatin treatment. We also identified a p73/p63-binding site in the promoter of the apoD gene that is responsive to the p53 family members. The ectopic expression of TAp73 as well as the addition of recombinant human apoD to culture medium induced the osteoblastic differentiation of the human osteosarcoma cell line Saos-2, as assessed by alkaline phosphatase activity. Importantly, apoD knockdown abrogated p73-mediated alkaline phosphatase induction. Moreover, TAp73-mediated apoD expression was able to induce morphological differentiation, as well as expression of neuronal markers, in the human neuroblastoma cell line SH-SY5Y. These results suggest that apoD induction may mediate the activity of p73 in normal development.

The p53 family consists of three members, p53, p73, and p63. These proteins share a high degree of amino acid sequence similarity and major functional domains. The p53 gene, the first member of the family to be identified, is the most frequent target gene for genetic alterations in human cancers. p53 protein is present at low levels in normal cells but is activated in response to environmental stimuli, such as DNA damage, hypoxia, viral infection, or oncogene activation, resulting in transactivation of a specific set of target genes. These targets are involved in cell cycle control, apoptosis, DNA replication, repair, proliferation, inhibition of angiogenesis, and cellular stress response (1–3). The other two members, p73 and p63, share high homology to p53, especially in the central DNA binding domain. Thus, p73 and p63 can bind to p53 response elements to transactivate a subset of p53 target genes and induce cell cycle arrest and apoptosis, which suggests that the p53 family members have a potential for functional overlap with p53 itself (4–8).

Despite a certain degree of overlapping functions, p73 and p63 have other activities that are different from p53. Unlike p53 as a classical tumor suppressor, p73 and p63 are rarely mutated in human cancers, suggesting that the two relatives are not classical tumor suppressor genes (9). Moreover, p73 and p63 encode multiple isoforms generated by the use of a second promoter or alternative 3′-end splicing. The TA2 isoforms act similarly to p53. In contrast, the ΔN isoforms lack the p53-like transactivation domain but do in fact retain transactivation activity. The ΔN isoforms are also thought to play a role in blocking transactivation of target genes of p53 and their respective TA isoforms. In general, therefore, the TA isoforms might have a role in tumor suppression, whereas increased expressed of the ΔN isoforms might be oncogenic (for review, see Refs. 9–12).

Moreover, studies of knock-out mice reveal an unexpected functional diversity among the p53 family genes. Flores et al. recently reported that p73−/−; p73−/−, and p63−/− animals die prematurely from tumors, suggesting a broader range of tumor suppressor functions for the p53 family than previously detected (13). p53-deficient mice develop normally (14), whereas p73-deficient mice exhibit neurological, phenomoral, and immune system defects (15). p63-deficient mice have major defects in their limbs and craniofacial development as well as a striking absence of stratified epithelia (16). In humans, heterozygous germ line mutations in the p63 gene are identified in patients with ectodactyly, ectodermal dysplasia, and facial clefts syndrome (17), suggesting that p63 plays an essential role in epidermal development during embryogenesis. Additionally,
p73α and p63α isoforms have a C-terminal region showing similarity with the sterile α motif (SAM) domain, a protein-protein interaction domain found in proteins involved in development regulation. Thus, these current data suggest that p73 and p63 are mainly involved in normal development and differentiation (for review, see Refs. 10–12). These differences among the p53 family probably depend on activation or repression of different sets of target genes.

In the context of this background, identifying the novel specific targets of p73 and p63 is an important step to better understand the roles of these genes in normal development. It also remains to be determined how different stimuli selectively recruit one or more members of the p53 family to achieve specialized transcriptional responses in specific cellular contexts.

Here, we report the identification of apolipoprotein D (apoD) as a direct transcriptional target of the p53 family members p73 and p63. ApoD is a 29-kDa glycoprotein primarily associated with high density lipoproteins in human plasma and is a member of the lipocalin family, based on its primary structure. Current evidence suggests that apoD plays an important role in the homeostasis or housekeeping functions of all organs. The apoD gene is expressed in many tissues, with high levels of expression in the spleen, testis, and brain. Although one of the functions of apoD is likely to be the transport of hydrophobic ligands, its physiological ligands are unknown. A marked up-regulation of apoD synthesis has been observed during nerve regeneration. ApoD may, therefore, participate in maintenance and repair within the central and peripheral nervous systems. Although its role in metabolism has yet to be defined, apoD is probably a multiple-ligand, multifunctional transporter (for review, see Refs. 18 and 19).

Chromatin immunoprecipitation and reporter assays demonstrated that TAp73 or TAp63 protein bind directly to the promoter region of the apoD gene, implying p73/p63-dependent transcriptional activity. We found that TAp73β-mediated apoD induction can enhance osteoblastic differentiation of human osteosarcoma cells and terminal differentiation of human neuroblastoma cells. Moreover, TAp73-mediated apoD expression was able to inhibit cancer cell proliferation. Taken together, our data reveal a role for TAp73 in the regulation of the apoD function involved in cellular differentiation and inhibition of cancer cell proliferation.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Recombinant Adenovirus**—The human cancer cell lines used in this study were purchased from the American Type Culture Collection or the Japanese Collection of Research Bioresources (Osaka, Japan). Mouse embryonic fibroblasts (MEFs) were prepared according to standard methods (20) from day 9.5 embryos and were maintained in DMEM containing 10% fetal calf serum. The generation, purification, and infection procedures of replication-deficient recombinant adenoviruses containing p53 (Ad-p53), TAp73α (Ad-p73α), TAp73β (Ad-p73β), TAp63α (Ad-p63α), and TAp63α (Ad-p63α) genes or the bacterial lacZ gene (Ad-lacZ) were described previously (21, 22). We titered adenovirus for the duplicate samples to confirm the reproducibility of the experiments. The relative efficiency of adenovirus infection was determined by 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) staining of cells infected with a control Ad-lacZ. 90–100% of the cells could be infected at a multiplicity of infection (MOI) of 25–100 (data not shown).

**Immunoblot Analysis**—The primary antibodies used for immunoblotting in this study are as follows: mouse anti-human p53 monoclonal antibody (mAb) (DO-1; Santa Cruz Biotechnology, Inc., Santa Cruz, CA); mouse anti-human p73 mAb (ER-15, Oncogene Research), 5B429 (Santa Cruz Biotechnology), and EP436Y (Epitomics); mouse anti-human p63 mAb (4A4, Oncogene Research); mouse anti-human p21 mAb (EA10, Oncogene Research); mouse anti-human apoD mAb (36C6, Novocasta); mouse anti-human GAP-43 mAb (GAP-7B10, Sigma); rabbit anti-human p27 polyclonal antibody (C-19; Santa Cruz Biotechnology); and mouse anti-human actin mAb (Chemicon). Whole cell lysates were prepared by scraping cell monolayers into radiolabile precipitation buffer without SDS (containing 150 mM NaCl, 50 mM Tris-HCl (pH 7.2), 1% deoxycholic acid, 1% Triton X-100, 0.25 mM EDTA (pH 8.0), protease and phosphatase inhibitors, 5 mg/ml leupeptin, 5 μg/ml aprotinin, 1 μg/ml pepstatin A, 1 μM phenylmethylsulfonyl fluoride, 5 mM NaF, and 100 μM sodium orthovanadate). Protein concentrations were quantitated (Lowry reagent; Bio-Rad). Equal protein quantities were separated by SDS-PAGE and transferred to Immobilon P membranes (Millipore).

**Microarray**—Total RNA was isolated from adenovirus-infected Saos-2 human osteosarcoma cells using TRIzol reagent (Invitrogen). U133 plus 2.0 GeneChip, which contains oligonucleotides representing 47,000 unique human transcripts, was purchased from Affymetrix. Experimental procedures were performed according to the GeneChip Expression Analysis Technical Manual. Washes and staining of the arrays were performed with an Affymetrix Fluidics Station 450, and images were obtained using an Affymetrix GeneChip Scanner 3000. Initial analysis and quality assessment of the array data were performed using GeneChip Operating Software version 1.1. Microarray data has been deposited into NCBI Gene Expression Omnibus (available on the World Wide Web) and are accessible through GEO series accession number GSE13504.

**Northern Blot and Semiquantitative RT-PCR**—For Northern blot analysis, total RNA (10 μg) was electrophoretically separated on a 1% agarose gel containing 2.2 M formaldehyde and blotted onto a nitrocellulose membrane (Schleicher & Schuell). RNA was visualized with ethidium bromide to ensure that it was intact and loaded in equal amounts and to confirm proper transfer. Hybridization was performed as described previously (23). cDNA probes for apoD (nucleotides 464–981) and p21 (nucleotides 11–429) were amplified by RT-PCR and were sequenced to verify their identity. For semiquantitative RT-PCR analysis, cDNAs were synthesized from 5 μg of total RNAs with the SuperScript preamplification system (Invitrogen). The RT-PCR exponential phase was determined within 20–30 cycles to allow semiquantitative comparisons among cDNAs from identical reactions. The PCR conditions involved an initial denaturation step at 94 °C for 2 min, followed by 30 cycles (for apoD) or 25 cycles (for GAPDH) of 94 °C for 30 s, 58 °C for 30 s, and 72 °C for 1 min. Oligonucleotide primer sequences were as follows: human TA-p73 sense (5’-CACCT-
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GGAGGGCATGACTAC-3', human ΔN/ΔN'p73 sense (5'-ACCATGCTGTACGTGCGTGAC-3'), human p73 antisense (5'-TTGCTTCCAGCGGATGAG-3'), mouse p73 sense (5'-GGAGCCTTGAGGTCTCTTAGG-3'), mouse p73 antisense (5'-GGTATGGAAAGGTAGTACGGC-3'), human apoD sense (5'-GGCAACTCTACTCATAGTAAA-3'), human apoD antisense (5'-GGGGCTGATGTACGTCGGTGAC-3'), mouse apoD sense (5'-CTTCCACACCGGAGGAG-3'), mouse apoD antisense (5'-CGGGCAGTGCTCGTGTAG-3'), human and mouse GAPDH sense (5'-ACACAGTCATCCATCCATC-3'), and human and mouse GAPDH antisense (5'-TCCACACCTGTGCTG-3'). The PCR products were visualized by electrophoresis on 1.5% agarose gels.

RNA Interference—Four human p73 siRNAs (si-p73-1 (5'-AAUUCUGCAUACCCUUGGGAG-3'), si-p73-2 (5'-AAAGACGUCAAUGCGAAUCCGUC-3'), si-p73-3 (5'-UUACACAGAAGUGUAAGCAGAUGG-3'), and si-p73-4 (5'-CAGAUCCCAUGAGCGGAAUdTdT-3')), human APOD siRNAs (5'-AAAUUCUCUCCACCGACGGAGAUGG-3', 5'-UCACUUCUGAUUGUAUCACUGAC-3', and 5'-AUAGUUCUCUAAGCCGGAGG-3'), two mouse p73 siRNAs (si-mp73-1 (5'-UGAGAUCUUGAGAAGUCAA) and si-mp73-2 (5'-CCCCUCUUGAGAAGUIGUA-3')), and nonspecific control siRNA (nonspecific control VII; target sequence 5'-NNACTCTATGCGCAGCTGAC-3') were purchased from Invitrogen or Qiagen and used according to the manufacturer's protocols. 1 × 10^5 cells were plated per well in 6-well plates with 2 ml of medium in each well. The following day, siRNAs were transfected using Oligofectamine (Invitrogen) to a final RNA concentration of 50 nM/well. Transfection was performed in triplicate and repeated at least three times.

Chromatin Immunoprecipitation (ChIP) Assay—ChIP assays were performed as described (23) using the ChIP assay kit (Upstate Biotechnology). 2 × 10^5 cells were cross-linked with a 1% formaldehyde solution for 15 min at 37 °C. The cells were then lysed in 200 μl of SDS lysis buffer and sonicated to generate 300–800-bp DNA fragments. After centrifugation, the supernatant was diluted 10-fold with the ChIP dilution buffer and split into three equal portions; one was incubated with the specific antibody (5 μg; DO-1 for p53, ER-15 for p73, and 4A4 for p63) at 4 °C for 16 h, and the other two portions were used as controls (anti-FLAG antibody and no antibody). One-fifth volume of total extract was used for PCR amplification as an input control. Immune complexes were precipitated, washed, and eluted as recommended. DNA-protein cross-links were reversed by heating at 65 °C for 4 h, and the DNA fragments were purified and dissolved in 40 ml of TE. Two microilters of each sample were used as a template. PCR amplification of the consensus p53-binding sequence upstream of the apoD gene (−419, renamed the RE-APOD) was performed on immunoprecipitated chromatin using the specific primers 5’-AGTCAGGAACTCTCATGCTC-3’ (forward) and 5’-CACAGCTGCTCCTCTGGAAG-3’ (reverse). Amplifications were also performed on immunoprecipitated chromatin using the specific primers 5’-CTTGGTGTGAC-GATCCTCTCCTTCTTCC-3’ and 5’-CTCACAATTCCTGAC-3’. The PCR products were visualized by electrophoresis on 1.5% agarose gels.

Luciferase Assay—A 40-bp fragment of the response element for p73, RE-APOD (5’-ATACCATATGTGGAAAATCATTGCTGAGCAGCTGGTCTG-3’) and its mutant form RE-APOD-mut (5’-ATACCATATGTGGAAAATCATTGCTGAGCAGCTGGTCTG-3’) were synthesized and inserted upstream of a minimal promoter in the pGL3-promoter vector (Promega), and the resulting constructs were designated pGL3-RE-APOD and pGL3-RE-APOD-mut, respectively. Subconfluent cultures in 24-well plates were transfected with 2 ng of pRL-TK reporter (Renilla luciferase for internal control) and 100 ng of pGL3 reporter (firefly luciferase, experimental reporter), together with 100 ng of a pcDNA3.1 control vector (Invitrogen) or a vector that expresses p53, TAp73α, TAp73β, or TAp63β by using Lipofectamine 2000 reagent (Invitrogen). Forty-eight hours after transfection, the reporter gene activities were measured by dual luciferase reporter assay (Promega), according to the manufacturer's instructions. All experiments were performed in triplicate and repeated at least three times.

Conditioned Medium—To obtain conditioned medium lacZ-CM and p73β-CM, SW480 cells were infected with Ad-LacZ and Ad-p73β at an MOI of 100, respectively. After 8 h, the medium was aspirated, and the cells were rinsed twice with serum-free DMEM. Fresh serum-free DMEM was then added, and cells were incubated for 16 h. Their conditioned media were collected and centrifuged to remove debris. For immunoblot analysis, the supernatant was concentrated using a Centri-10 concentrator (Millipore).

ALP Activity—ALP activity was determined using p-nitrophenyl phosphate as a substrate (24). Total cell lysates or culture medium were assayed for ALP activity using LabAssay ALP (Nakalai Tesque, Kyoto, Japan). Enzyme activity assay was performed in assay buffer containing 6.7 mM p-nitrophenyl phosphate as a substrate. The reaction was stopped by adding 0.2 M NaOH, and absorbance was read on an optical densitometer at 405 nm using a plate reader. Relative ALP activity is defined as mmol of p-nitrophenyl phosphate hydrolyzed/min per mg of total protein or per ml of medium.

Differentiation of Neuroblastoma Cells—To assess the differentiated phenotype in vitro, SH-SY5Y cells (1 × 10^5 cells) were seeded into 12-well plates. After 24 h, cells were incubated with lacZ-CM or p73β-CM with 0.5% fetal calf serum. Recombinant human apoD protein (200 ng/ml; Abcam) was added to the medium of some cultures. The conditioned medium was renewed every 24 h for up to 3 days, cells were then examined under a ×40 objective lens, and images were recorded using a CCD camera VB-7000 (Keyence, Osaka, Japan). Cell differentiation was also assessed by immunoblot analysis of GAP-43 and p27.

Colony Formation Assay—For colony formation assays, 5 × 10^3 cells were plated in triplicate in 60-mm tissue culture plates. After 16 h, the cells were incubated with the conditioned...
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medium lacZ-CM or p73β-CM for 48 h. When necessary, recombinant human apoD protein (100 ng/ml) was added to the medium. After 2 weeks, colonies were fixed and stained. The percentage of colony survival was determined from the number of colonies in the control group. The assay was repeated in at least three independent experiments.

Statistical Analysis—Experimental data were evaluated using Student’s t test, with probability values less than 0.05 considered significant.

RESULTS

TAp73 and TAp63 Induce Expression of ApoD mRNA and Protein—To express the human p53 family genes, we generated replication-deficient recombinant adenoviral vectors Ad-p53, Ad-p73α, Ad-p73β, Ad-p63γ, and Ad-p63α for the p53, TAp73α, TAp73β, TAp63γ, and TAp63α genes, respectively. To determine the relative efficiency of adenovirus-mediated gene transfer, cells were infected with adenovirus containing the bacterial lacZ gene (Ad-lacZ). We used several human cancer cell lines that showed highly efficient gene transfer, with 90–100% of the cells staining for β-galactosidase activity at an MOI of 25–100. Infection with Ad-p53, Ad-p73α, Ad-p73β, Ad-p63γ, and Ad-p63α resulted in expression of exogenous p53, TAp73α, TAp73β, TAp63γ, and TAp63α proteins, respectively (e.g. see Fig. 1A). We mainly used TAp73β and TAp63γ isoforms in this study, because we and others have demonstrated that transcription of a p53 response reporter gene was activated more strongly in TAp73β than in TAp73α and more strongly in TAp63γ than TAp63α, respectively (5, 21, 22). In an effort to identify targets specifically regulated by p73, we performed microarray analysis and compared expression patterns in a human osteosarcoma cell line, Saos-2, infected separately with Ad-p53 and Ad-p73β (data not shown). Using this approach, we found that the apoD gene was reproducibly up-regulated at least 4-fold in p73-transfected cells relative to p53-transfected cells. Northern blot analysis demonstrated that expression of the apoD gene was increased considerably in a time-dependent manner in A172 and DLD1 cells by infection with Ad-p73β but was not significantly induced by infection with Ad-p53 (Fig. 1B). The apoD induction was observed as early as 12 h after Ad-p73β infection. In contrast, the p21 gene was induced by p53 as well as by its family members (Fig. 1B). Moreover, we examined the effect of induction of p73 in five human cancer cell lines, including A172, SW480 (colorectal cancer), Saos-2 (osteosarcoma), U373 (glioma), and MKN28 (stomach cancer). The status of the endogenous p53 gene in these lines is mutant for SW480, DLD1, and MKN28; wild type for A172; and null for Saos2. As shown in Fig. 1C, apoD mRNA was clearly induced by TAp73β in all cell lines tested but not significantly by p53. Although apoD was still induced in response to TAp73α in four of the five cell lines tested (A172, SW480, U373, and MKN28), the degree of induction was less remarkable than that of the TAp73β isoform. In contrast, p53 induced p21 significantly in all cell lines tested (Fig. 1C, middle).

To determine whether the increase in apoD mRNA was accompanied by an increase in protein expression, we then examined the level of apoD protein by immunoblot using an antibody against human apoD. Fig. 2A shows increased total cellular apoD protein in p73-transfected cells. Furthermore, an ~30-kDa protein was detected by immunoblotting with antibodies against human apoD in a CM used for SW480 cells.
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FIGURE 2. Induction of apoD protein by p53 family members in human cancer cell lines. A, cells were infected with adenovirus as described above and harvested 24 h after infection. Immunoblot analysis was performed using an anti-apoD antibody. B, secreted apoD protein in conditioned media. 2 × 10^6 SW480 cells were infected with adenovirus at an MOI of 100, as described above. After 8 h, the medium was aspirated, and the cells were rinsed twice with serum-free DMEM. Fresh serum-free DMEM was then added, cells were incubated for 16 h, and the media were collected and centrifuged to remove debris. The supernatant was concentrated for immunoblot analysis. The blot was probed with an anti-apoD antibody.

ApoD Expression Is Regulated by Endogenous p73—The effect of p53 family members on apoD induction shown above was based on their overexpression. We then addressed whether this effect could occur under more physiological conditions in noncancerous HEK293 cells using double-stranded RNA oligonucleotides (siRNA) corresponding to a part of the p73 cDNA sequence. p73 siRNAs used here were designed to target all p73 isoforms. An siRNA lacking sequence homology with any human or mouse gene was used as a control siRNA (si-cont). The TA isoforms of p73 were prominently expressed in HEK293 cells (Fig. 3A). HEK293 cells were transfected with p73 siRNAs every 24 h for three consecutive days. Both mRNA and protein levels of p73 were significantly reduced in cells transfected with p73 siRNAs (Fig. 3A). We then evaluated the expression of apoD after treatment with p73 siRNAs. apoD transcript levels were significantly reduced in the p73-silenced HEK293 cells when compared with mock- or si-cont-transfected cells (Fig. 3A). Similar results were observed for MEF cells. A specific reduction in p73 mRNA was detected in MEF cells transfected with p73 siRNAs (Fig. 3B). The apoD mRNA level was also significantly reduced in p73-silenced MEF cells when compared with mock- or si-cont-transfected cells. These findings indicated that transcription of the apoD gene is regulated by p73 in normal mouse cells as well as human cancer cells.

Additionally, we used cisplatin, a DNA-damaging agent known to activate endogenous p73 through transcriptional activation and protein stabilization (25–28). As seen in Fig. 3C, endogenous TAp73 protein increased in SW480 cells following exposure to 10 μM cisplatin for 24 h.
RT-PCR analysis showed a parallel increase of apoD mRNA following cisplatin treatment (Fig. 3C, fifth panel). To further validate that the apoD mRNA increase after cisplatin treatment was dependent on TAp73, we down-regulated endogenous p73 by siRNA. Transfection of each of four p73 siRNAs separately in SW480 cells in vitro strongly inhibited the accumulation of TAp73 protein following cisplatin treatment without affecting the protein levels of β-actin (Fig. 3C, CDDP (+)). Notably, p73 siRNAs, but not untreated (siRNA (−)) or control siRNA (si-cont), inhibited basal expression as well as induction of apoD mRNA after cisplatin treatment (Fig. 3C). We also analyzed the endogenous mRNA expression of T and ΔN isoforms of p73 using semiquantitative RT-PCR (Fig. 3C, fifth and bottom panels; TAp73 and ΔN/ΔN′p73). The DN/ΔN′p73 mRNA levels were generally low in human cancer cells (supplemental Fig. 1). mRNA levels of ΔNp73, a dominant negative isoform, were also low in SW480 cell lines. These results show that activation of endogenous TAp73 following cisplatin treatment mediates induction of the apoD gene.

Identification of a Specific Binding Sequence for TAp73 and TAp63 in the apoD Gene—To determine whether apoD is a direct target of transcriptional activation by TAp73 and TAp63, we searched for consensus p53-binding sequences in the genomic locus encoding human apoD. Using this approach, we found only one putative p53-binding site within 10 kb around exon 1 of the apoD gene. This candidate sequence was identified at nucleotide position −419, where +1 represents the transcription start site (Fig. 4A). To determine whether the TAp73 and TAp63 proteins could bind to this candidate site in vivo, we performed a ChIP assay using SW480 cells infected with Ad-p53, Ad-p73β, and Ad-p63y. Immunoprecipitation of DNA-protein complex using antibodies against p53, p73, and p63 was performed on formaldehyde-cross-linked extract from Ad-p53−, Ad-p73β−, and Ad-p63y−infected SW480 cells, respectively. We then measured the abundance of this candidate sequence within the immunoprecipitated complexes by PCR. As shown in Fig. 4B, DNA fragment containing this candidate at −419 was reproducibly present in the immunoprecipitated complex containing p73 or p63 protein (second panel, lanes 7 and 10). We designated this sequence RE-APOD (for responsive element in apoD), which consists of three copies of the consensus 10-bp motif each separated by 5-bp (Fig. 4A). Nucleotide sequence comparison revealed that the RE-APOD sequence is well conserved between human and mouse at a specific side effect of the siRNA treatment. These results strongly suggest that TAp73 induces the osteoblastic differentiation of the human osteosarcoma Saos-2 cells mainly via transactivation of the apoD gene.

p73 Induces Osteoblastic Differentiation via apoD Transactivation—To explore the physiological function of apoD as a mediator of TAp73, we designed siRNAs targeting human apoD and a nonspecific siRNA (si-cont) as a control and introduced them separately into Saos-2 cells every 24 h for 2 consecutive days. Pretreatment of Saos-2 cells with apoD siRNAs strongly inhibited apoD protein accumulation following TAp73β overexpression (Fig. 5A, compare lane 2 with lane 4). ALP is a useful marker of relatively early osteoblast differentiation. To clarify whether apoD mediates the function of the p73 in normal development, we then examined ALP activity. The transfection was performed as described above. At 8 h after the last transfection of siRNA, ALP activity was measured using total cell lysates or conditioned media. When si-cont-transfected Saos-2 cells were infected with Ad-p73β, the ALP activity was increased by 1.4-fold (cell lysates) and 5.2-fold (medium). The treatment with recombinant apoD resulted in increase in the ALP activity as well. Importantly, treatment with apoD siRNA inhibited p73-mediated ALP induction in Saos-2 cells (Fig. 5B, lane 5). The addition of exogenous recombinant apoD rescued the effect of apoD siRNA (Fig. 5B, lane 6), unambiguously showing that the effect observed is indeed due to down-regulation of apoD expression and not due to a nonspecific side effect of the siRNA treatment. These results strongly suggest that TAp73 induces the osteoblastic differentiation of the human osteosarcoma Saos-2 cells mainly via transactivation of the apoD gene.

p73 Induces Terminal Differentiation in Neuroblastoma Cells via apoD Transactivation—To assess the role of apoD in p73-mediated neuronal differentiation, we prepared conditioned medium from cultures of Ad-p73β-infected SW480 cells (p73β-CM). Fig. 6A shows the increase in apoD protein in the
FIGURE 4. A response element for p73 and p63 in the apoD gene. A, the position and nucleotide sequence of a p73/p63 response element, RE-APOD. Open boxes, exons. RE-APOD is located 419-bp upstream of the transcription start site and consists of three copies of the consensus 10-bp motif of the p53-binding sequence. The consensus sequences are indicated by capital letters, and lowercase letters identify mismatches with the consensus sequence. The spacer sequences between the 10-bp motifs are underlined. Alignment of the conserved p73/p63 binding sites in mouse sequences from the apoD gene is shown at the top. A mutated sequence corresponding to potentially critical nucleotides of RE-APOD used in the luciferase assay is also indicated (RE-APOD-mut). R, purine; Y, pyrimidine; W, adenine or thymine. B, TAp73 and TAp63 proteins bind to the RE-APOD site in vivo. ChIP assay of a genomic fragment (nucleotide positions -419 to -380, where +1 represents the transcription initiation site) containing the RE-APOD site in Ad-p53-infected (lanes 4–6), Ad-p73β-infected (lanes 7–9), and Ad-p63α-infected SW480 cells (lanes 10–12) is shown (second panel). Immunoprecipitation was performed using a gene-specific antibody against p53 (lane 4), p73 (lane 7), and p63 (lane 10), followed by PCR amplification. Immunoprecipitates with an anti-FLAG antibody (lanes 5, 8, and 11) served as negative controls. Approximately 1% of the input chromatin samples (lanes 1–3) and 10% of the ChIP samples (lanes 4–12) were used as templates in each PCR. PCR amplification revealed that a similar amount of MDM2 promoter sequence is present in p53, p73, and p63 complexes extracted from each immunoprecipitate (first panel). The DNA fragment containing the RE-APOD sequence was amplified in the samples immunoprecipitated with an antibody against p73 and p63 (second panel). A genomic fragment containing no p53 consensus binding sequences within intron 2 was amplified in the input control but not in the immunoprecipitated material (third panel). C, the RE-APOD sequence is responsive to TAp73 and TAp63. H1299, HEK293, and SW480 cells were transiently transfected with the pGL3-promoter vector containing the RE-APOD or its mutant version (RE-APOD-mut) along with pRL-TK using Lipofectamine 2000 reagent. Cells were cotransfected with a control pcDNA3.1 vector or a vector that expresses p53, TAp73α, TAp73β, and TAp63α 24 h prior to performing the luciferase assay. Luciferase activity was measured using the dual-luciferase reporter assay system with the Renilla luciferase activity as an internal control. All experiments were performed in triplicate, and the mean and S.D. is indicated by the bars and brackets, respectively. D, regulation of apoD transcription by endogenous p73. ChIP assay for the presence of p73 protein at RE-APOD was carried out on untreated (CDDP (-); lanes 4 and 5) and cisplatin-treated (CDDP (+); lanes 6 and 7) SW480 (first panel) and Saos-2 cells (third panel). DW, a no-template control (lane 1).
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**FIGURE 5.** p73 induces alkaline phosphatase activity in Saos-2 cells via apoD transactivation. A, Saos-2 cells were transfected with control (lanes 1 and 2) or apoD siRNA (lanes 3 and 4) every 24 h for 2 consecutive days. Eight hours before the last transfection of siRNA, cells were infected with Ad-lacZ (lanes 1 and 3) or Ad-p73β (lanes 2 and 4) at an MOI of 50. Eighteen hours after the last transfection of siRNA, expression of the indicated protein was determined by immunoblotting. B, the transfection was performed as described above. At 8 h after the last transfection of siRNA, ALP activity was measured using total cell lysates or conditioned media as described under “Experimental Procedures.” A proportion of the cells were treated with recombinant human apoD (100 ng/ml) 16 h before measurement, indicated as rhAPOD (+). The results represent the means ± S.D. of quadruplicate determinations. *, p < 0.05; **, p < 0.01, Student’s t test.

**FIGURE 6.** p73 induces neuronal differentiation of SH-SY5Y cells via apoD transactivation. A, SW480 cells were transfected with control (lanes 1 and 2) or apoD siRNA (lanes 3 and 4) every 24 h for 2 consecutive days. Cells were infected with Ad-lacZ (lanes 1 and 3) or Ad-p73β (lanes 2 and 4) at an MOI of 100, 8 h before the last siRNA transfection. After 8 h, the cells were rinsed twice with serum-free medium. Fresh serum-free medium was then added, and cells were incubated for 16 h. CM was collected and centrifuged to remove debris. The supernatant was concentrated for immunoblot analysis. The blot was probed with an anti-apoD antibody. B, SH-SY5Y cells were incubated in the indicated CM for 3 days. Recombinant human apoD (200 ng/ml) was added to the medium of some cultures (lane 4, rhAPOD (+)). Cell lysates were used for immunoblotting and probed with antibodies specific for GAP-43, p27, and actin. C, representative images of SH-SY5Y cells grown in the indicated CM are shown.

Conditioned medium from p73β-infected SW480 cells. Pretreatment of SW480 cells with apoD siRNAs (si-APOD) but not with a nonspecific siRNA (si-cont) strongly inhibited the accumulation of apoD in the medium of Ad-p73β-infected cells (Fig. 6A, compare lane 2 with lane 4). We then cultured neuroblastoma SH-SY5Y cells in conditioned medium containing 0.5% fetal bovine serum and evaluated the expression of the neuronal marker proteins GAP-43 (29) and p27 (30). GAP-43 and p27 protein levels were significantly increased when SH-SY5Y cells were cultured with (p73β/si-cont)-CM for 3 days (Fig. 6B, lane 2) compared with cells cultured with (lacZ/si-cont)-CM (Fig. 6B, lane 1). In contrast, p73β-mediated GAP-43 and p27 induction was attenuated when the p73β-infected cells were pretreated with apoD siRNA (Fig. 6B, lane 3). Importantly, the addition of recombinant apoD to the conditioned medium rescued the effect of apoD siRNA (Fig. 6B, compare lane 3 with lane 4). We then analyzed the morphological differentiation of SH-SY5Y cells cultured with p73β-CM. After 3 days of treatment with p73β-CM, SH-SY5Y cells developed phenotypic changes typical of neuronal morphology characterized by neurite outgrowth (29, 30) (Fig. 6C). These morphological signs of SH-SY5Y differentiation were inhibited when the p73β-infected cells were pretreated with apoD siRNA (Fig. 6C, (p73β/si-APOD)-CM). Moreover, the addition of recombinant human apoD to the conditioned medium rescued the effect of the apoD siRNA (Fig. 6C, (p73β/si-APOD)-CM + rhAPOD). These results strongly suggest that TAp73 induces the neuronal differentiation of SH-SY5Y cells primarily through transactivation of the apoD gene.

ApoD Induced by p73 Suppresses Cancer Cell Proliferation—The inverse relationship between apoD expression and cell proliferation has also been found in breast and prostate cancer cell lines (31). We performed a colony formation assay to examine whether apoD expression could contribute to the proliferation-suppressing effect of p73 on cancer cells. We prepared a conditioned medium used for culturing Ad-p73β-infected SW480 cells and then cultured HCT116 and HeLa cells in this medium (p73β-CM). The conditioned medium from p73β-infected SW480 cells. Pretreatment of SW480 cells with apoD siRNAs (si-APOD) but not with a nonspecific siRNA (si-cont) strongly inhibited the accumulation of apoD in the medium of Ad-p73β-infected cells (Fig. 6A, compare lane 2 with lane 4). We then cultured neuroblastoma SH-SY5Y cells in conditioned medium containing 0.5% fetal bovine serum and evaluated the expression of the neuronal marker proteins GAP-43 (29) and p27 (30). GAP-43 and p27 protein levels were significantly increased when SH-SY5Y cells were cultured with (p73β/si-cont)-CM for 3 days (Fig. 6B, lane 2) compared with cells cultured with (lacZ/si-cont)-CM (Fig. 6B, lane 1). In contrast, p73β-mediated GAP-43 and p27 induction was attenuated when the p73β-infected cells were pretreated with apoD siRNA (Fig. 6B, lane 3). Importantly, the addition of recombinant apoD to the conditioned medium rescued the effect of apoD siRNA (Fig. 6B, compare lane 3 with lane 4). We then analyzed the morphological differentiation of SH-SY5Y cells cultured with p73β-CM. After 3 days of treatment with p73β-CM, SH-SY5Y cells developed phenotypic changes typical of neuronal morphology characterized by neurite outgrowth (29, 30) (Fig. 6C). These morphological signs of SH-SY5Y differentiation were inhibited when the p73β-infected cells were pretreated with apoD siRNA (Fig. 6C, (p73β/si-APOD)-CM). Moreover, the addition of recombinant human apoD to the conditioned medium rescued the effect of the apoD siRNA (Fig. 6C, (p73β/si-APOD)-CM + rhAPOD). These results strongly suggest that TAp73 induces the neuronal differentiation of SH-SY5Y cells primarily through transactivation of the apoD gene.

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The primary results from this study demonstrated that endogenous TAp73-mediated apod secretion is able to inhibit cancer cell proliferation (supplemental Fig. 2). Taken together, these results strongly support our conclusion that apoD, a secretory molecule positively regulated by TAp73, functions as a cell proliferation-inhibitory factor.

**DISCUSSION**

The primary results from this study demonstrated that endogenous as well as exogenous TAp73 regulated apoD gene transcription. Northern blot and immunoblot analysis revealed that apoD expression levels increase dramatically and rapidly in response to overexpression of TAp73 (Figs. 1 and 2). ApoD transcript levels were significantly reduced in MEFs and HEK293 cells after p73 siRNA transfection (Fig. 3A). Additionally, we showed that cisplatin treatment induced apoD mRNA in an endogenous p73-dependent manner (Fig. 3B). ApoD induction by exogenous TAp63γ was less dramatic but occurred in five of the six cell lines tested (no induction in U373 cells). Activation of apoD by TAp63 and TAp73 appears to occur independently of p53 status, since both TAp63 and TAp73 can activate the apoD in cells either mutant or wild-type for p53. In contrast, apoD was not significantly induced by p53 overexpression in the same condition in which p21 was activated in each of the cell lines (Figs. 1 and 2). Thus, our findings clearly indicate that apoD is a direct target for the p53 family members, especially for TAp73.

We also found a specific binding site for TAp73 and TAp63 proteins, RE-APOD, in the human apoD gene by a ChIP assay (Fig. 4B). A reporter assay demonstrated that RE-APOD is important for p73/p63-dependent transactivation (Fig. 4C). The RE-APOD sequence consists of three copies of the 10-bp p53-binding motif separated by two 5-bp spacer sequences, whereas nearly all p53 response elements reported previously contain two adjacent copies of the 10-bp motif (1–4, 32, 33). Our laboratory and others have reported that response elements for p73 and p63 are found in Aquaporin-3 (34), the p73 gene itself (35), Jagged1 (23), IL-4Rα (36), PEDF (37), Sonic Hedgehog (38), and flotillin-2 (39) genes and consist of three or more copies of the 10-bp motif separated by spacer sequences. Therefore, spacing between at least three copies of the 10-bp motif may be important for determining the binding specificity of the p53 family member proteins. Consistent with our data, p53 and p63γ differentially activate a luciferase reporter gene under the control of the response elements with a 1-nucleotide spacer between the two 10-bp motifs having three mismatches from the consensus sequence (40). A comparison of the human and mouse apoD genes revealed that this transactivation is mediated by the conserved RE-APOD sequence upstream of the transcription start site (Fig. 4). Moreover, apoD transcript levels were significantly reduced in p73-silencing MEFs when compared with mock or untreated cells, suggesting that TAp73 is also essential for endogenous apoD expression in mouse cells. Therefore, we concluded that the apoD gene is a direct and evolutionarily conserved transcriptional target of the p53 family member genes. On the other hand, apoD gene expression is known to be up-regulated in cultured human cancer cells, fibroblasts, and MEFs when the cells undergo growth arrest (41, 42). De Carmo et al. (42) examined functional analysis of the
apoD promoter and found that the region between nucleotides −588 and −179 is responsible for the induction of apoD expression following growth arrest (wherein position 1 corresponds to the transcription start site). RE-APOD, a specific binding site for TAp73 and TAp63 proteins, is located within this region, suggesting that p53 family members are implicated in the growth arrest-induced apoD gene expression.

The tumor suppressor p53 is critically important in the cellular DNA damage response. Two members of the mammalian p53 family, p73 and p63, encode proteins that share considerable structural homology with p53, suggesting that the p53 family genes have a potential for functional overlap. Indeed, the expression of several p53-regulated genes can also be induced by p73 and p63, although recent studies show a marked divergence in the developmental roles of p63 and p73 and further distinguish these p53 family members from p53. Thus, target genes of p53 family members have been proposed to be divided into at least two categories. The first category includes genes that are activated by all of the p53 family genes and broadly involved in growth arrest and apoptosis (43, 44). This category includes p21, MDM2, 14-3-3σ, puma, etc. The cell context, the type of stimulus, and the quantitative balance between the diverse p53 family members could be some of the key determinants in dictating the final biological output. In the second category, target genes are selectively activated by each of the p53 family members, comprising p53, p73, p63, and their related isoforms, and mediate a specific biological activity. The present study indicates that apoD might fall in the second category of genes. Our results also suggest that the two p53 family proteins could play a role distinct from p53 by inducing specific target genes.

p53 family members, especially p73, are involved in neuronal and epidermal differentiation. apoD is a component of the plasma lipid transport system, and its expression was previously shown to occur in senescent fibroblast cultures and in subsets of neurons and glia during neural development (41, 45–47). We therefore tested whether apoD expression mediated by p73 could induce cellular differentiation of osteosarcoma cells by means of activity of ALP, a marker of relatively early osteoblast differentiation. Ectopic expression of TAp73β as well as the addition of recombinant apoD resulted in an increase in the ALP activity in Saos-2 cells. This effect of TAp73β on the ALP activation was abrogated by the addition of apoD siRNA. In this study, we examined the neurotrophic activity of TAp73-induced apoD expression in SH-SY5Y cells, a well known in vitro differentiation model. As judged by neurite extension and the increased expression of neuronal marker proteins, apoD expression significantly contributed to the neuronal differentiation of SH-SY5Y cells induced by TAp73. The present results suggest an association between p53 relatives and apoD, which is not shared by p53, and therefore raise the possibility that the p53 family members play a role in normal development in part through apoD induction. Since p73 plays a pivotal role during development in mice (10), in particular during the development of the nervous system (48), and in neuronal maintenance and survival in the mature CNS (49, 50), the p73-directed regulation of apoD provides a potential mechanism by which p73 could participate in cellular differentiation.

ApoD expression also correlates inversely with aggressive behavior of several different types of malignant tumors (51–54). In addition to its role in cell differentiation, our study provides a novel link between TAp73-mediated apoD induction and cancer cell proliferation. We showed that TAp73 expression resulted in an increase in secrerion of apoD protein into the culture medium. This increase in apoD secretion was associated with an inhibition of cancer cell proliferation in vitro. Importantly, the effect of TAp73 on proliferation suppression was partially blocked by pretreatment with apoD siRNA (Fig. 7). These studies demonstrate that an essential role of TAp73 in growth suppression of cancer cells is mediated, in part, by apoD induction and its action as a paracrine factor. Although the underlying mechanism has not yet been elucidated, apoD has recently been shown to function in cell growth inhibition (55–58). Additionally, Lopez-Boado et al. (31) found that induction of apoD expression by retinoic acid is accompanied by a decrease in cell proliferation and an increase in cell differentiation in a human breast cancer cell line. Thus, our data suggest that p73-induced apoD expression inhibits cancer cell proliferation by inducing cell cycle arrest and/or cell differentiation.

Gene replacement of the wild-type p53 gene has been pursued as a potential gene therapy strategy in several types of cancer (59). This strategy potentially relies on p53-mediated apoptosis, which in turn depends on induction of a distinct class of transcriptional target genes (60). On the other hand, death of tumor cells modified with the wild-type p53 gene may also lead to the killing of uninfected neighboring tumor cells through a bystander effect (61–63). Our laboratory and others have reported a significant anti-tumor effect following TAp73 overexpression, as assessed in animal models of gene therapy as well as in cultured cells (21, 22, 64, 65). Thus, similar to p53, TAp73 may induce bystander effects via apoD induction.

In conclusion, we have found that apoD is a direct downstream target of p53 family member genes. Our results suggest a role of TAp73 in the regulation of apoD involved in cellular differentiation and inhibition of cancer cell growth. In vertebrates, apoD is secreted from subsets of neurons and glia during neural development and aging (45, 46, 66). Recently, GLaz, a fly homolog of apoD, was found to have a protective role in stress situations, and its absence reduces life span and accelerates neurodegeneration (67, 68). The role of apoD as an effector of p73 during neural development and aging should be explored in future studies. Additionally, apoD is a member of the lipocalin superfamily of transporter proteins and a component of human plasma high density lipoproteins. Thus, further characterization of the role of p53 family members and apoD in lipid metabolism is an important area for future research, with possible therapeutic implications for treatment of metabolic disorders, such as diabetes and obesity.
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