

# Possible Oncogenic Potential of $\Delta$ Np73: A Newly Identified Isoform of Human p73<sup>1</sup>

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## Abstract

**p73, a recently identified gene highly homologous to p53, can transactivate p53 target genes and induce apoptosis. Here we report the identification of an NH<sub>2</sub>-terminal truncated isoform of human p73,  $\Delta$ Np73, which is capable of suppressing p53- and p73-dependent transactivation. We speculate that this suppression is achieved by competing for the DNA binding site in the case of p53 and by direct association in the case of TAp73. Expression of  $\Delta$ Np73 in cancer cell lines also inhibited suppressive activity of p53 and TAp73 in colony formation, implying possible involvement of  $\Delta$ Np73 in oncogenesis by inhibiting the tumor-suppressive function of p53 and TAp73. Also reported is the identification of TAp73 $\eta$ , a new member of the COOH-terminal truncated isoform of p73 and tissue-specific expression of these isoforms, along with other previously identified p73 isoforms.**

## Introduction

p53, a tumor suppressor, is a critical gene in deciding the destiny of cells with DNA damage. Genotoxic stress causing DNA lesions will lead to marked accumulation and activation of the p53 gene products, liberating its intrinsic transactivator function. As a result, it is believed to induce cellular G<sub>1</sub> arrest increasing the chance for a successful DNA repair process or apoptosis in irreparable cells. The loss of functional p53 then results in accumulation of premalignant cells with irreversible genetic damage, so that its mutations are most frequently found among human tumors (1).

This p53 has long been a lone gene with no related genes until the recent successive identification of the new family members, p73 and p51/p63 (also known as p40, p73L, CUSP, or KET; Refs. 2–5). In addition to a structural similarity to p53, they both resemble this gene functionally, at least in *in vitro* overexpression systems. They are capable of transactivating p53 target genes, inducing apoptosis, and suppressing cell growth to various extents, depending on the individual isoform (Refs. 2–7; see below). Despite this resemblance, they were shown to exhibit distinct biological functions; p51-targeted mice suffered from severe defects in the development of skin and extremities; p51 mutations were shown to play a causative role in human hereditary diseases such as EEC (ectrodactyly, ectodermal dysplasia, and facial clefts), dactylaplasia, and Hay-Wells syndromes (2); and p73-targeted mice exhibited defects in neuronal development and inflammatory responses (8). Furthermore, p73 and p51 was transcribed as numerous splicing variants, which are expressed in a tissue specific manner (4–8).

In an attempt to delineate this complex pattern of expression, our first effort was devoted to determine the genomic structures for these

p53 family genes. Careful genomic analysis of human p73 and p51 enabled us to predict the presence of an alternative promoter and exon of p73 similar to those of p51. This exon encoded NH<sub>2</sub>-terminal truncated isoforms of p73 corresponding to the p51 isoforms termed  $\Delta$ Np51/p63 (5), which was shown to have characteristic potential to suppress transcriptional activity of both p53 and TAp51/p63. During analyses of the isoform, the discovery and functional evaluation of murine  $\Delta$ Np73 were described (8, 9). We report here the first identification of human  $\Delta$ Np73. We show that it has the potential to suppress p53 or TAp73, implying that  $\Delta$ Np73 may function as an oncogenic isoform. Along with the analyses of  $\Delta$ Np73, we also describe the discovery of a COOH-terminal truncated variant of p73, TAp73 $\eta$ , and analyses of the tissue-specific expression pattern of p73 isoforms in detail.

## Materials and Methods

**Cell Culture and Luciferase Assay.** Human lung carcinoma cell line H1299 (p53-null) was cultivated in RPMI 1640, and human osteosarcoma cell line SaOS-2 (p53-null) and the simian COS7 cell line were cultivated in DMEM supplemented with 10% fetal bovine serum in 5% CO<sub>2</sub> at 37°C. Transient transfection of SaOS-2 cells and measurement of luciferase activity were carried out as described (4). The p53 expression construct containing arginine at residue 72 and mdm2-P2 promoter reporter plasmid were gifts from Drs. B. Vogelstein (Howard Hughes Medical Institute and Johns Hopkins Oncology Center, Johns Hopkins University School of Medicine, Baltimore, MD) and M. Oren (Department of Molecular Cell Biology, Weizmann Institute of Science, Rehovot, Israel), respectively.

**Construction of a Panel of cDNA Libraries and Semiquantitative PCR.** We constructed a human cDNA library panel using 33 human poly(A)+ RNAs purchased from Clontech (Palo Alto, CA) using the SuperScript Lambda System for cDNA synthesis and  $\lambda$  cloning (Life Technologies, Inc., Rockville, MD), essentially as suggested by the supplier. Expression of the NH<sub>2</sub>-terminal splice variants of the p73;TA,  $\Delta$ N', and  $\Delta$ N was detected by semiquantitative PCR. TA and  $\Delta$ N' was detected by use of 5'-GGAATAATGAGGTGGTGGGC-3' designed from exon 3 and 5'-GCCTGTTTACAAGAAAGCGG-3' designed from exon 5.  $\Delta$ N was detected by use of 5'-GCGAAAATGCCAA-CAAACGG-3' designed from exon 3' and the above exon 5 primer. PCR for the COOH-terminal variants of p73  $\alpha$ ,  $\beta$ ,  $\gamma$ ,  $\delta$ ,  $\epsilon$ , and  $\zeta$  was performed using the sense primer designed from exon 10 (5'-GAAGCTGAAAGAGAGC-CTGG-3') and the antisense primer designed from exon 14 (5'-GATGGT-CATGCGGTACTGC-3'). The expected length of the amplified fragments for the  $\alpha$ ,  $\beta$ ,  $\gamma$ ,  $\delta$ ,  $\epsilon$ , and  $\zeta$  was 517, 423, 367, 190, 273, and 230 bp, respectively. To detect  $\eta$ , the sense primer designed from exon 11 (5'-CCTTCTCTCCT-TGCTCTCG-3') and the antisense primer designed from exon 13 (5'-TTCTCGCCCATGAACAAGG-3') were used. PCR reaction was done by use of Taq DNA polymerase (Life Technologies, Inc., Rockville, MD) and anti-Taq start antibody (Clontech) as suggested by the suppliers.

**Cloning of  $\Delta$ Np73 $\alpha$ / $\beta$  and TAp73 $\eta$ .** A DNA fragment including p73 exon 3' was PCR amplified using the above exon 3 primer and an antisense primer designed from exon 6 (5'-GTGATGATGATGAGGATGGG-3') and then cloned into pRcCMV (Invitrogen, Carlsbad, CA) to obtain  $\Delta$ Np73 $\alpha$ / $\beta$  expression plasmids. HA<sup>3</sup>-tagged and FLAG-tagged constructs were made by addition of synthetic oligonucleotides encoding the HA epitope of MYPD-

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<sup>3</sup> The abbreviations used are: HA, hemagglutinin antigen; RACE, rapid amplification of cDNA ends; MOI, multiplicity of infection.

VPDYA and the FLAG epitope of MDYKDDDDK to the NH<sub>2</sub>-terminal of TAp73/ $\Delta$ Np73. A DNA fragment containing the TAp73 $\eta$  isoform amplified by the 3'-RACE method was combined with TAp73/pRcCMV to create an expression construct.

**Immunoprecipitation and Immunoblot Analysis.** Cells were transiently transfected with 10  $\mu$ g of DNA per 10-cm dish and harvested after 48 h. Cell lysate preparation and immunoprecipitation were carried out as described either using anti-p53 (Oncogene Research Products, Cambridge, MA), anti-HA (Boehringer Mannheim, Tokyo, Japan), or anti-FLAG (Sigma Chemical Co.-Aldrich, Tokyo, Japan) antibody (10). The immunoprecipitants were resolved by SDS-polyacrylamide gels and immunoblotted with the corresponding antibodies.

**Colony Formation and Cell Proliferation Assay.** The indicated expression plasmids were cotransfected with pBABEpuro, a puromycin-resistance plasmid (a gift from J. Morgenstern) into H1299 cells using Lipofectamine (Life Technologies, Inc., Rockville, MD) as described (4). H1299 stable transfectants were isolated by transfecting  $\Delta$ Np73 $\alpha$  or pRcCMV using Lipofectamine with pBABEpuro as a selection plasmid. The recombinant adenovirus expressing human TAp73 $\alpha$  cDNA (Ad-TAp73 $\alpha$ ) and LacZ (Ad-LacZ) were constructed as described (11). Cell viability was assayed by alamarBlue (Alamar Biosciences, Sacramento, CA).

## Results

**Identification of Two New Isoforms of Human p73:  $\Delta$ Np73 and TAp73 $\eta$ .** Analyses of the genomic structures of *p73* and *p51/p63* done by us and others revealed striking overall structural resemblance between the two, most noticeably conservation of lengthy introns 1 and 3 and remaining short introns (12). Furthermore, careful comparison between the two led us to predict a putative exon between exons 3 and 4 in the *p73* gene, which may encode the  $\Delta$ Np73 isoform, corresponding to  $\Delta$ Np51. Naturally, our next attempt was to isolate unidentified isoforms by an efficient RACE method (to be described elsewhere), which enabled us to isolate  $\Delta$ Np73 corresponding to  $\Delta$ Np51 lacking an NH<sub>2</sub>-terminal transactivation domain. Comparison of the  $\Delta$ Np73 cDNA to its genomic sequence revealed the existence of exon 3' in intron 3 of *p73* gene. The length of the *p73* intron 3' (between exon 3' and exon 4) was approximately 15 kb, which was similar to that of the *p51* gene. Another interesting feature is the two-way usage of this exon 3', which might also be operating in the case of *p51*, considering the striking resemblance (Fig. 1A). One usage is the exon 3' transcribed by an alternative promoter similar to that of  $\Delta$ Np51. The 5'-RACE method showed that *p73* has 276 bases of exon 3', which is homologous to that of murine *p73* and similar to that of *p51* (8). The other usage is that the exon 3' is being transcribed along with exon 3 by an alternative splicing event. This was determined by PCR screening of human cDNA libraries using various primers. This latter transcript,  $\Delta$ N'p73 mRNA, was shown to retain all of the other exons 1 through 14 with 198 base-exon 3' insertion. Both  $\Delta$ Np73 and  $\Delta$ N'p73 mRNA encoded an identical protein with a truncated NH<sub>2</sub>-terminal structure lacking the putative transactivation domain of TAp73 proteins (Fig. 1B). In addition to this 5'-RACE method, the 3'-RACE method enabled us to obtain a new COOH-terminal truncated isoform of p73, termed TAp73 $\eta$ . Other than the two originally identified isoforms of p73, p73 $\alpha$  and p73 $\beta$  (generated by exon 13 skipping), four splicing variants of p73 have since been reported: p73 $\gamma$ ,  $\delta$ ,  $\zeta$ , and  $\epsilon$  generated by skipping the exon of 11, exons of 11–13, exons 11 and 12, and exons 11 and 13, respectively. Contrasting with these isoforms, p73 $\eta$  is generated by alternative termination (Fig. 1A). p73 $\eta$  mRNA has a long exon 13 (853 bp) containing a stop codon, encoding a 571-amino acid protein (Fig. 1C). As shown in Fig. 1A, there are 11 different p73 mRNAs: TAp73 $\alpha$ , TAp73 $\beta$ , TAp73 $\gamma$ , TAp73 $\delta$ , TAp73 $\epsilon$ , TAp73 $\zeta$ , TAp73 $\eta$ ,  $\Delta$ Np73 $\alpha$ ,  $\Delta$ Np73 $\beta$ ,  $\Delta$ N'p73 $\alpha$ , and  $\Delta$ N'p73 $\beta$ .

## Expression Pattern of p73 Isoforms in Normal Human Tissues.

Our cDNA library system is also useful for determining a tissue-specific expression profile by performing semiquantitative PCR. Using a set of human cDNA library panels, normalized for transferrin receptor and glyceraldehyde-3-phosphate dehydrogenase expression, we semiquantitatively determined the expression pattern of various isoforms of human p73. PCR primers were designed so as to distinguish individual isoforms (see "Materials and Methods"). TAp73 and  $\Delta$ N'p73 transcripts were distinguishable by the product length, 642 and 444 bp, respectively. The expression of the latter transcript was solely detected in the pancreas, whereas many tissues were positive for TAp73 expression. The  $\Delta$ Np73 transcript was only detected in fetal lung and corpus callosum by conducting PCR in separate reactions amplifying the 606-bp product (Fig. 1D). p73 $\alpha$  was expressed in a wide variety of human tissues, whereas p73 $\beta$  was expressed in lymph nodes, spinal cord, cerebellum, and substantia nigra; p73 $\gamma$  was expressed in liver, small intestine, fetal lung, cerebellum, and thalamus (Fig. 1D). Only one 200-bp product was amplified in the hippocampus, which could be either p73 $\delta$ ,  $\epsilon$ ,  $\zeta$ , or a mixture of these (Fig. 1D). The p73 $\eta$  transcript was only detected in the lymph node (data not shown). Although not as potent, TAp73 $\eta$  showed significant transactivation potential comparable with that of p51A/TAp63 $\gamma$ , TAp73 $\alpha$ , and TAp73 $\gamma$  against mdm2-P2 (Fig. 2A), BAX, p21waf1, 14-3-3 $\delta$ , and RGC promoters (data not shown).

**Inhibition of TAp73- and p53-dependent Transactivation by  $\Delta$ Np73.** We investigated the potential of the  $\Delta$ N isoforms in inhibiting p53 or TAp73 transactivation activity. A designated amount of  $\Delta$ Np73 plasmid was cotransfected into SaOS2 cells with a constant amount of either p53 or TAp73 expression plasmid. FLAG tagged  $\Delta$ Np73 $\alpha$  and  $\beta$  almost completely suppressed the transactivation of mdm2-P2 promoter by TAp73 $\beta$  and TAp73 $\alpha$  (Fig. 2B and similar data not shown). Similarly, transactivation of p53 was suppressed by the FLAG- $\Delta$ Np73 $\alpha$  and  $\beta$ , although a four times larger amount was required to suppress the transactivation of p53 effectively (Fig. 2C). The expression constructs without the FLAG tag were capable of suppressing p53 and TAp73 transactivation to the same extent (data not shown).

## Determination of Interaction between $\Delta$ Np73 with Either p53 or TAp73.

To determine the mechanism of the above suppression, we investigated the ability of the  $\Delta$ Np73 proteins to bind to either p53 or TAp73. Lysates prepared from HA-TAp73 ( $\alpha$  or  $\beta$ ) and FLAG- $\Delta$ Np73 ( $\alpha$  or  $\beta$ ) cotransfectants were subjected to immunoprecipitation by an anti-FLAG monoclonal antibody and then immunoblot detection by an anti-HA monoclonal antibody (Fig. 3, A and B). The experiment revealed readily detectable interaction of HA-TAp73 ( $\alpha$  or  $\beta$ ) and FLAG- $\Delta$ Np73 ( $\alpha$  or  $\beta$ ). The reciprocal experiment using tagged epitopes and antibodies *vice versa* resulted in essentially identical results (data not shown). On the other hand, p53 was not coimmunoprecipitated with FLAG- $\Delta$ Np73 $\alpha$  or  $\beta$  (Fig. 3C) under the same conditions used, indicating that p53 does not stably interact with p73. We also experienced reduction of FLAG- $\Delta$ Np73 in the presence of p53 (Fig. 3C). This is speculated to be caused by the general inhibitory effect of p53 on promoter activity (13) or by enhanced  $\Delta$ Np73 degradation mediated by p53, as was observed in the case of  $\Delta$ Np63 (14).

**Effect of  $\Delta$ Np73 on Cell Growth.** On the basis of the above observations of suppressive potential of  $\Delta$ Np73, we next examined the effect of  $\Delta$ Np73 expression on cell growth- inhibitory activity of p53 and TAp73 by colony formation assay. Cotransfection of  $\Delta$ Np73 $\alpha$  with either TAp73 $\alpha$  or with p53 resulted in significant or dramatic increment in the number of colonies compared with transfection of either alone (Fig. 4A). Although suppression of colony formation by TAp73 $\alpha$  expression had a marginal effect with no significant differ-

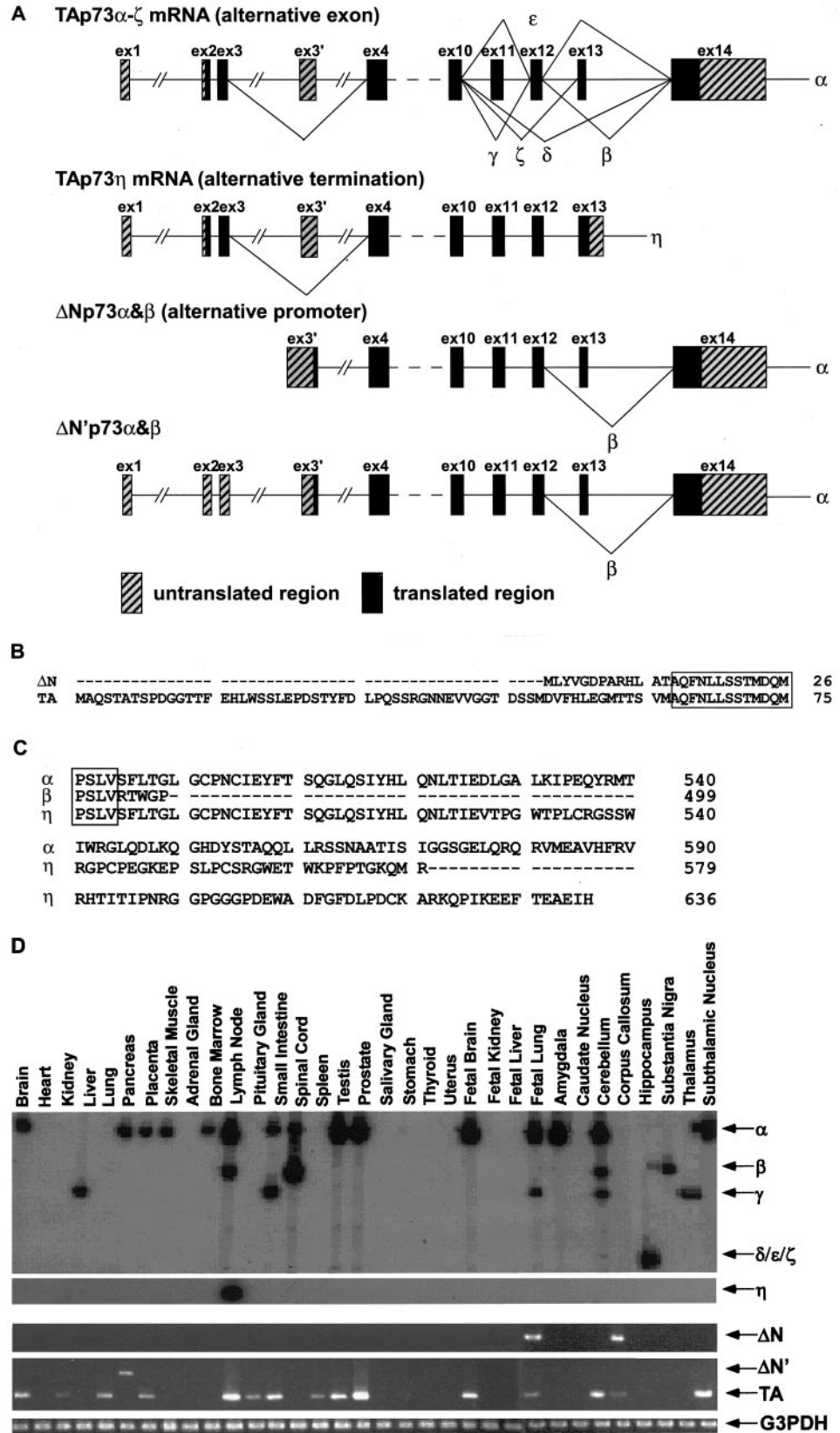


Fig. 1. p73 isoforms and their expression. A, schematic representation of p73 genomic structures responsible for generating various isoform transcripts. The hatched boxes show the 5' and 3' untranslated regions, and the closed boxes indicate the translated regions. Note that  $\Delta$ Np73 has two types of mRNA in the 3'-untranslated region by two-way usage of exon 3',  $\Delta$ Np73 and  $\Delta$ N'p73. Nevertheless, they both encode an identical protein,  $\Delta$ Np73. B, alignment of the NH<sub>2</sub>-terminal amino acid sequence of  $\Delta$ Np73 and TAp73. C, alignment of the COOH-terminal amino acid sequence of TAp73 $\eta$  and others. D, expression pattern of various p73 isoforms in human tissues. The COOH-terminal splice variants (upper two panels) and the NH<sub>2</sub>-terminal splice variants (lower two panels) of p73 were semiquantitatively detected by performing PCR using cDNA libraries constructed from 33 different human tissues as templates. ex, exon.

ence from pRcCMV alone, cotransfection of  $\Delta$ Np73 $\alpha$  plasmid apparently increased the number of colonies. Thus, the assay revealed the potential of  $\Delta$ Np73, which can be interpreted as either anti-growth suppressing or growth promoting.

Then, we established two cell clones, H1299 cell clones stably expressing FLAG- $\Delta$ Np73 $\alpha$  (H1299- $\Delta$ Np73 $\alpha$ ) and stably integrating pRcCMV (H1299-RcCMV). H1299- $\Delta$ Np73 $\alpha$  cells, expressing exogenous  $\Delta$ Np73 $\alpha$ , exhibited more rapid growth profile than did H1299-

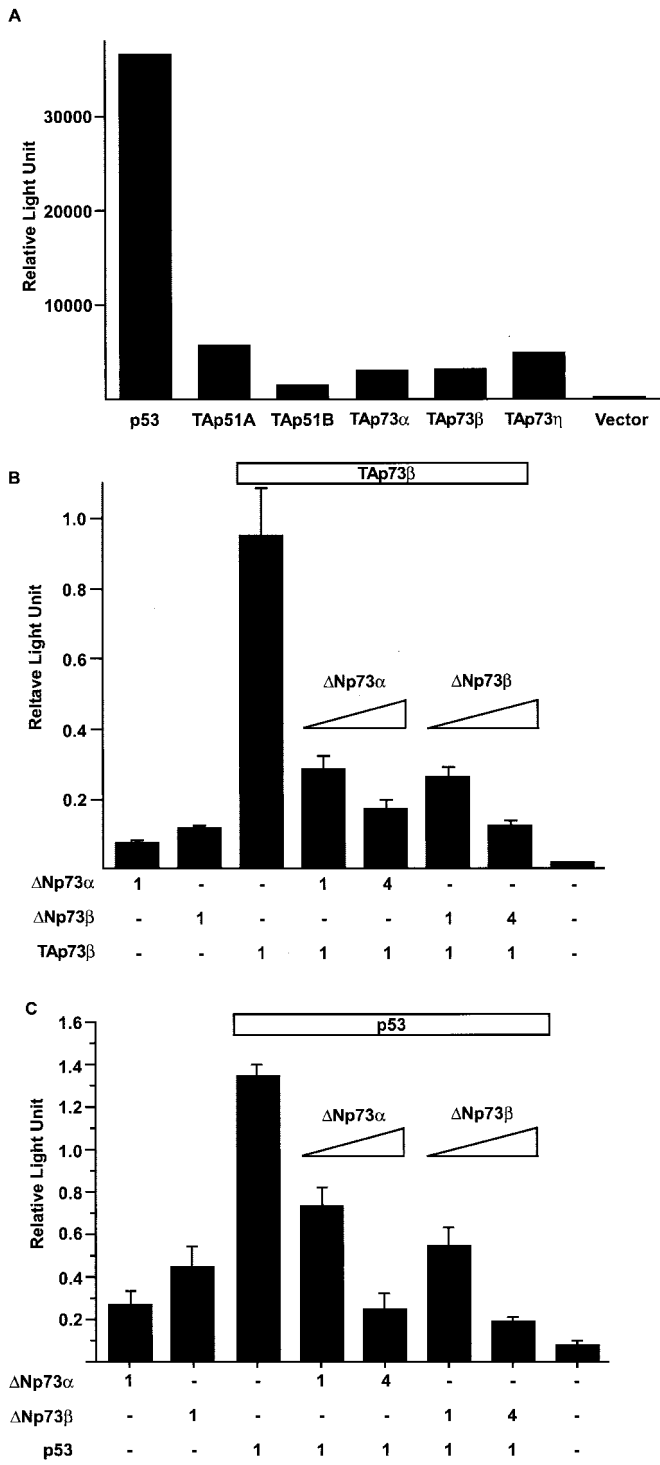


Fig. 2. A, transactivation potentials of various p73 isoforms. p53, TAp51A, TAp51B, TAp73α, TAp73γ, TAp73η, expression plasmids or empty pRcCMV vector was cotransfected into SaOS-2 cells with mdm2-P2 promoter-luciferase plasmids and internal control plasmids (pRL-CMV). Luciferase activity was measured by a Dual Luciferase assay kit. B, effects of ΔNp73 expression on transactivation activity of TAp73β. FLAG-tagged ΔNp73α or ΔNp73β was cotransfected into SaOS2 cells at the ratios of 1:0, 0:1, 1:1, and 1:4 with respect to TAp73β with mdm2-P2 promoter-luciferase plasmids and internal control plasmids (pRL-TK). C, effects of ΔNp73 expression on transactivation activity of p53. p53 was used instead of TAp73β in B.

RcCMV cells, when infected with Ad-TAp73α at the MOI of 100. In contrast, the growth rates of these two clones when infected with Ad-LacZ at the similar MOI resulted in almost identical profiles (Fig. 4B). Independent experiments using 20 and 50 MOI of Ad-TAp73α or

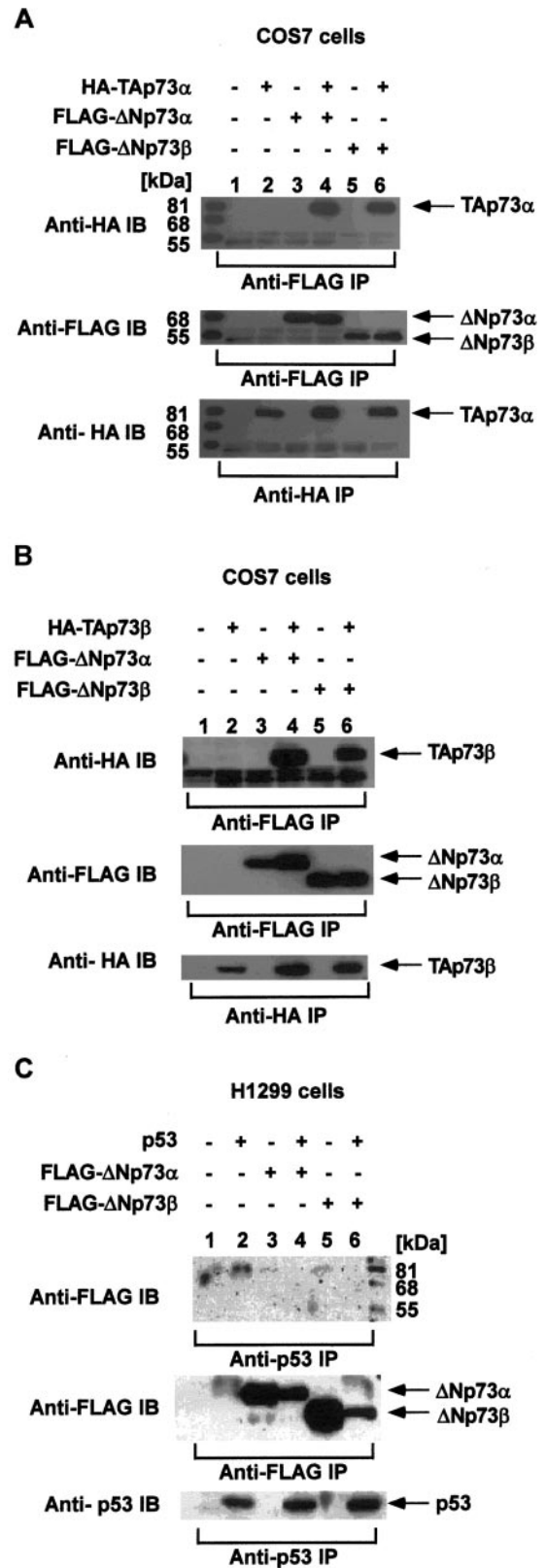


Fig. 3. A, interaction between TAp73α with ΔNp73. COS7 cells were cotransfected with HA-tagged TAp73α and either FLAG-tagged ΔNp73α or ΔNp73β as indicated. Lysates prepared from the transfectants were immunoprecipitated with anti-HA monoclonal antibody and then subjected to Western blot detection with anti-FLAG monoclonal antibody. B, interaction between TAp73β with ΔNp73. Analyses similar to A were done, using HA-tagged TAp73β instead of HA-tagged TAp73α. C, interaction between p53 with ΔNp73. Analyses similar to A were done, using p53 expressing plasmid, FLAG-tagged ΔNp73α, or ΔNp73β and H1299 cells as transfectants.

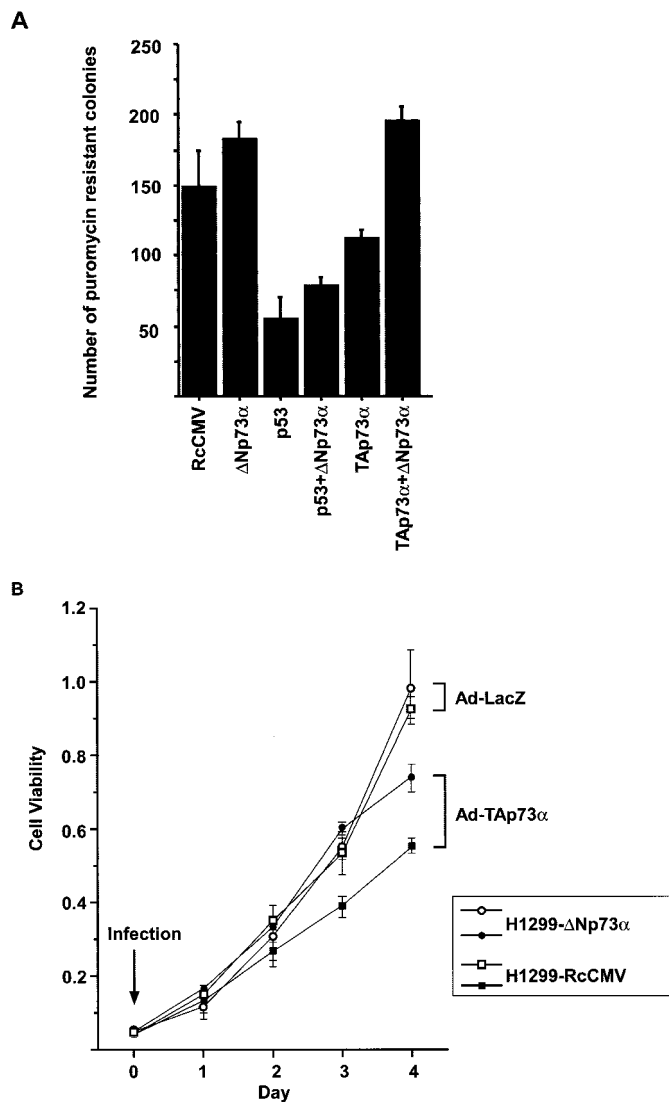


Fig. 4. A, effects of  $\Delta$ Np73 $\alpha$  expression on colony formation. The indicated expression constructs were cotransfected with pBABEpuro into H1299 cells. After 2 weeks of puromycin selection, the number of colonies was counted. The average numbers of colonies of three independent experiments are shown; bars, SE. B, effects of  $\Delta$ Np73 $\alpha$  expression on H1299 cell growth. H1299 cells ( $1 \times 10^3$ /well) stably expressing  $\Delta$ Np73 $\alpha$  (H1299- $\Delta$ Np73 $\alpha$ ; ●, ○) or transfected with empty pRcCMV vector (H1299-RcCMV; ■, □) were selected. These cells were plated 1 day before adenoviral infection. Days were counted starting from day 0 when Ad-TAp73 $\alpha$  (●, ■) or Ad-LacZ (○, □) were infected. Bars, SE.

Ad-LacZ confirmed the resistance of H1299- $\Delta$ Np73 $\alpha$  cells (data not shown). These data suggest that the cells expressing  $\Delta$ Np73 $\alpha$  are more resistant to the growth-inhibitory effect of TAp73 $\alpha$  and p53. Nevertheless, the results regarding TAp73 $\alpha$  were somewhat weak because of the fact that TAp73 $\alpha$  did not suppress cell growth to the extent reported previously by others (6, 7).

## Discussion

The genomic structures of *p73* and *p51/p63* were more similar to each other than either of the two to *p53* gene in the following aspects: (a) *p73* and *p51* have a huge intron 1 (>30 kb) and intron 3 (>15 kb); (b) both encode various isoforms by exon skipping: *p73* $\beta$ ,  $\gamma$ ,  $\delta$ ,  $\epsilon$ ,  $\zeta$ , and *p63* $\beta$ ; (c) both possess an alternative promoter producing transcript encoding  $\Delta$ N isoforms; and (d) *p73* encoded the isoform TAp73 $\eta$  by alternative termination, and *p51* encoded the isoform *p51A/p63* $\gamma$  by alternative exon (Fig. 1A). As a result of this complex

genomic structure common to these two genes, they exhibited a much more complex pattern of expression than that of *p53*. Furthermore, we speculate the existence of different regulations among different species. Contrasting with predominant expression of  $\Delta$ Np73 in the murine brain as reported (8), we only detected human  $\Delta$ Np73 expression in the corpus callosum among eight subregions of the brain. In addition, neither TA nor  $\Delta$ N isoform of human *p73* was expressed in hippocampus, whereas *p73*-deficient mice were demonstrated to suffer from hippocampal dysgenesis (8).

In addition to *mdm2* oncoprotein, a well-known negative regulator of *p53* and *p73*, we discovered a novel mechanism of regulation of *p73* and possibly *p53* by  $\Delta$ N isoforms. It is of note that the amount of  $\Delta$ Np73 comparable with that of TAp73 was fully capable of suppressing the transactivation by TAp73, whereas a 4-fold larger amount was necessary to suppress *p53* to the same extent (Fig. 2, B and C).  $\Delta$ Np73 bound to TAp73 but was incapable of binding to *p53* *in vivo* (Fig. 3), which was consistent with the data showing that wild-type *p53* cannot associate with TAp73 in mammalian cells (15–17). Thus, we speculate that the suppression of TAp73 transactivation was achieved by direct association, whereas the suppression of *p53* was achieved by competition for the DNA binding element.

One reason that the *p53* mutations are the most frequently encountered in human tumors is attributable to the nature common to the vast majority of *p53* mutant proteins, *i.e.*, they are capable of inhibiting the remaining wild-type *p53* allele in a dominant-negative manner (1). In contrast, the mutation of the *p73* gene in human tumor is reported to be rare, despite the structural and functional similarity to *p53* (18, 19). Nevertheless, our analyses gave rise to the possibility that the overexpression of  $\Delta$ Np73 may contribute to the genesis of tumors by negating the tumor-suppressive activity of *p53* or TAp73. In fact, overexpression of *p73* in some tumors of the lung, bladder, and liver has been described (18). Although the overexpressed isoform of *p73* has not been determined for most of these cases, it is highly likely that they are the  $\Delta$ Np73 isoform. Furthermore, one of the  $\Delta$ N isoforms of the *p51* gene, *p40*, is reported to be oncogenic (20). It can readily be interpreted that the overexpression of the  $\Delta$ N isoform is much more easily actualized than acquisition of point mutations as in the case of *p53*. Further investigation is under way, and we are currently searching for human tumors with  $\Delta$ Np73 overexpression.

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