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The expression of the ΔNp73β isoform of p73 leads to tetraploidy

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ABSTRACT

The p73 locus gene has a complex structure encoding a plethora of isoforms. The different ΔN truncated isoforms of p73 may exert different activities depending on the cellular context. The β isoform of ΔNp73 seems to have a particular pattern of action even if its role in cell cycle and mitosis is still under investigation. To gain further knowledge of ΔNp73β's function, we investigated the effects of its over-expression in tumour cellular models, using the tetracycline-inducible expression system. In the human lung carcinoma cell line H1299, ΔNp73β over-expression resulted in suppression of cell growth and in cell death. Surprisingly stable over-expression of ΔNp73β impaired the genomic stability of tumour cells, leading to the formation of tetraploid cells. The cells become enlarged and multinucleate, with incorrect mitotic figures, and died by apoptotic-independent pathways. Our data suggest that ΔNp73β-induced aberrant mitosis evades the control of the mitotic spindle assay checkpoint, leading to tetraploidy and cell death through mitotic catastrophe rather than apoptosis. The various C-terminal regions of ΔNp73 may influence the final cellular phenotype and we assume that the β one in particular could be important in both cell growth control and regulation of mitosis.

1. Introduction

TP53 is the prototype tumour suppressor gene in human cancers on account of its vital role in deciding the destiny of cells in response to cellular stresses and DNA damage. Inactivation of the p53 pathway occurs in more than half of human sporadic cancers, and germline mutation of p53 results in Li-Fraumeni syndrome.[1,2]

For almost two decades, p53 was considered unique in its structure and function, but recently the discovery of two TP53-related genes, TP73 and TP63, derived probably from a common ancestor, has outlined a new gene family with similar, but not overlapping, functions.[3] p53 and p73 share architectural homologies in their typical structure of transcription factors, with an N-terminal transactivation (TA) domain, a central DNA binding domain (DBD) and a C-terminal oligomerisation domain (OD). In addition, these genes express an array of isoforms predicting a complex set of transcription.[4] Despite these similarities, they have different functions. p53 is an ubiquitous protein mostly regulated at the post-transcriptional level and strongly specialised in stress response, while p73 is a tissue-specific protein regulated both at the

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transcriptional and at the post-transcriptional level with roles both in response to genotoxic stress and in epithelial and neurological differentiation.[5]

The TP73 gene (1p36,33) gives rise to various N- and C-terminal isoforms generated through either alternative exon splicing or using a second promoter. The N-terminal p73 isoforms can be grouped in two classes: the transactivation-competent TAp73 proteins and the transactivation-defective, N-terminally truncated ΔTAp73 proteins (ΔN'p73, Ex2p73, Ex2/3p73 and ΔNp73).[5] The ΔNp73 isoforms lack 62 residues that constitute the TA domain, but possess 13 unique residues in their N-terminus which constitute a novel TA domain.[6] Despite the differences in N- and C-termini, all p73 isoforms conserve the DBD and two N- and one C-terminal proline-rich motifs (PXXP motif), which are indispensable for the TA function.[6,7]

The TAp73 isoform mimics p53, inducing cell cycle arrest and apoptosis in response to DNA damage.[8–10] However, it also transactivates a unique set of target genes,[8–10] suggesting that it has a distinct role from p53. TAp73 also appears to play a physiological role in the conclusive stages (late anaphase/telophase) of mitosis because the abrogation of TAp73 expression affects mitotic completion and exit to the subsequent interphase.[11,12] These findings suggest that TAp73 also has an important function in cell growth control.

The function of ΔNp73 isoforms is still controversial since in specific circumstances they may behave differentially. Early studies provided evidence that ΔNp73, lacking the TA domain, was transcriptionally inactive and acted as a dominant negative regulator of both wild type p53 and TAp73, inhibiting their pro-apoptotic effect in response to chemotherapeutic agents through hetero-oligomerisation with p73 or p53, or competitive inhibition in p53 binding to its DNA responsive elements.[13,14] It was also reported that ΔNp73 promotes immortalisation in primary fibroblasts and cooperates with the oncogene Ras to drive their transformation in *in vitro* and *in vivo*.[15,16] Thus, in primary human tumours ΔNp73 may display some 'oncogenic' features. On the other hand, recent studies suggest that ΔNp73α and β may be regulators of signal transduction because they influence the expression of various genes in a p53-independent fashion (despite the lack of the TA domain).[6,17,18] In addition, in both p53 null and wild type cells, over-production of ΔNp73α does not seem to affect cell growth or response to chemotherapy,[19–21] while over-expression of ΔNp73β suppressed cell growth.

These data suggest that ΔNp73 isoforms may have variable or even some opposing biological functions in different cellular contexts and that the various C-terminal regions of ΔNp73 may influence the final cellular phenotype.[22] The evidence that cells have multiple p73 isoforms with multiple activities and that there is a functional cross-talk among all family members, endowing them with both tumour suppressor and oncogenic roles, ruled out the notion that all these variables need to be considered in defining the role of p53 family genes in tumourigenesis and cell responses to genotoxic stresses.

Among all C-terminal ΔNp73 variants, we studied the role of ΔNp73β isoform in regulating the cell cycle in tumour cell models. *In vitro* ΔNp73β over-expression induces mitotic defects and impinges the genomic stability of tumour cells, leading to tetraploidy. Additionally, we found that the tetraploid status was associated with correct S-phase entry but the suppression of entry in subsequent mitosis.

2. Materials and methods

2.1. Cell culture

The H1299 cell line (human lung carcinoma $p53^{-/-}$) was routinely maintained in RPMI1640 medium supplemented with 10% foetal calf serum (FCS).

We generated different stable ΔNp73β over-expressing clones using a tetracycline-regulated expression system (T-Rex System, Invitrogen). Briefly, ΔNp73β cDNA (kindly provided by Dr. De Laurenzi) was subcloned in the tetracycline-inducible plasmid pcDNA4/TO and was used to transfect a H1299 clone expressing the tetracycline repressor (pcDNA6/TR). Clones were screened for the inducible expression of the ΔNp73β and two of them (H1299/ΔNβ18 and H1299/ΔNβ20) were selected to grow in medium supplemented with 10% TET System-approved foetal bovine serum (BD biosciences) with 5 μg/mL of blasticidin (InvivoGen) and 10 μg/mL zeocin (Invitrogen). We used the H1299/Mock clone transfected with the pcDNA4/TO empty vector as internal control. All cells were grown at 37 °C in a 5% CO_2 atmosphere.

2.2. Real time RT-PCR

Real time RT-PCR was used for the relative quantification of ΔNp73. Three hundred nanograms of total RNA purified with the SV40 Total RNA Isolation System (Promega) were retrotranscribed in 20 μl with Archive Kit (Applied Biosystem) and 2 μl further amplified by *Real Time* PCR (ABI Prism 7900 Sequence Detection System, Applied Biosystem). Primers and probe sequences to detect the levels of ΔNp73 were 5'-GGATTCCAGCATGGACGTCTT-3' as forward primer and 5'-CGCCTACCATGCTGTACGT-3' as reverse primer and 5'-GGCTGCTCATCTGGTCCAT-3' as TaqMan probe (Assay by Design, Applied Biosystems). Primers and TaqMan probe sequences to detect the actin mRNA levels were supplied as ready-to-use solution (Assay on Demand, Applied Biosystems). Reactions were run in a total volume of 25 μL with TaqMan PCR Master Mix, following the manufacturer's instructions (Applied Biosystems).

2.3. Western blot

For Western blot analysis, doxy-untreated and treated cells were washed twice with ice-cold PBS, removed by scraping and collected by centrifugation. Cells were lysed in ice-cold lysis buffer (50 mM tris pH8, 150 mM NaCl, 1 mM EGTA, 100 mM NaF, 10% glycerol, 1 mM $MgCl_2$ and 1% Triton X-100) containing protease inhibitors (Sigma) and incubated on ice for 30 min. Samples were centrifuged at 13,000x*g* for 10 min at 4 °C and the protein content of the supernatant was determined using a Bio-Rad Protein assay (Bio-Rad).

Forty micrograms of total cellular protein was resolved by SDS-PAGE on an 8% polyacrylamide gel and electrotransferred to PVDF membrane. Immunoblot analysis was done using the following antibodies: rabbit anti-p73 (1:1000, Oncogene