p73 and p63 Sustain Cellular Growth by Transcriptional Activation of Cell Cycle Progression Genes

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Abstract

Despite extensive studies on the role of tumor suppressor p53 protein and its homologues, p73 and p63, following their overexpression or cellular stress, very little is known about the regulation of the three proteins in cells during physiologic cell cycle progression. We report a role for p73 and p63 in supporting cellular proliferation through the transcriptional activation of the genes involved in G_1-S and G_2-M progression. We found that in MCF-7 cells, p73 and p63, but not p53, are modulated during the cell cycle with a peak in S phase, and their silencing determines a significant suppression of proliferation compared with the control. Chromatin immunoprecipitation analysis shows that in cycling cells, p73 and p63 are bound to the p53-responsive elements (RE) present in the regulatory region of cell cycle progression genes. On the contrary, when the cells are arrested in G_0-G_1, p73 detaches from the REs and it is replaced by p53, which represses the expression of these genes. When the cells move in S phase, p73 is recruited again and p53 is displaced or is weakly bound to the REs. These data open new possibilities for understanding the involvement of p73 and p63 in cancer. The elevated concentrations of p73 and p63 found in many cancers could cause the aberrant activation of cell growth progression genes and therefore contribute to cancer initiation or progression under certain conditions. [Cancer Res 2009;69(22):OF1–9]

Introduction

The p53 tumor suppressor protein plays a critical role in cell responses to several stressors, in cell cycle checkpoints, and in the maintenance of genomic integrity, whereas the p53-related proteins p63 and p73 are mainly involved in differentiation and development (1, 2).

p53 and *p73* genes express multiple spliced COOH-terminal and NH2-terminal variants, whereas p63 gene expresses at least three alternatively spliced COOH-terminal isoforms (TA isoforms). Moreover, all three genes could be transcribed from an alternative promoter located in intron 3 for *p73* and *p63*, and in intron 4 for *p53*. This leads to the expression of NH2-terminally truncated isoforms (ΔN isoforms), which exert dominant-negative effects on the activity of TA isoforms; in addition, it has been shown that they directly activate specific target genes and have antiapoptotic and proproliferative potential (2).

Despite extensive studies on the activity of p53, p73, and p63 isoforms following their overexpression, the regulation and the functions of the three proteins during cell cycle progression under physiologic conditions are unknown.

The P1p73 promoter is a target of the transcription factor E2F1 at the G_1-S transition, which leads to the accumulation of TAp73 proteins during S phase (3, 4). In late S phase, TAp73 proteins are then phosphorylated by cyclin-dependent kinases on Thr86 and their transcriptional activity is inhibited (5–7).

p53 activity is also modulated during the cell cycle by posttranslational modifications. At the G_1-S transition, p53 is phosphorylated on Ser315 by the cyclin A/cyclin-dependent kinase 2 complex. In this state, p53 is bound by E2F factors and is therefore retained in the nucleus where it remains active. When cells enter S phase and the level of cyclin A increases, because the E2F/cyclin A interaction is favored against E2F/p53 interaction, Ser315 phosphorylated p53 is no longer retained in the nucleus and, being translocated in the cytoplasm, it becomes inactive (8). A role for the ΔNp53 isoform (also named p47 or Δ40p53) in regulating cell cycle progression has also been suggested on the basis of its accumulation during progression in S phase after serum stimulation (9). On the other hand, it has been shown that ΔNp53 has a role in modifying p53 cell localization and in inhibiting p53 degradation by MDM2 (10).

Little is known on p63 modulation during cell cycle progression. Therefore, we have aimed to investigate, in a comprehensive study, the regulation and the functions of p53 family members during physiologic cell cycle progression.

Materials and Methods

Cell cultures. The human lung carcinoma H1299 and the human breast carcinoma MCF-7 cell lines were cultured in DMEM plus 10% fetal bovine serum.

Cell proliferation assays by MTT reduction and bromodeoxyuridine incorporation. MCF-7 cells were transfected with 80 nmol/L of scramble siRNA or specific siRNA against p53, TAp73, ΔNp73, TAp63, ΔNp63, pcDNA3 control vector, or pcDNA3-p53, -TAp73α, -ΔNp73α, -TAp63α, and -ΔNp63α expression vectors. After 72 h, 200 μL of MTT solution (5 mg/mL) was added to the cells which were incubated for 4 h at 37°C. Then the supernatant was removed and the blue formazan crystals were resuspended in isopropanol prior to reading the absorbance at 580 nm. The bromodeoxyuridine incorporation was determined by using Cell Proliferation ELISA bromodeoxyuridine (Roche) as described by the manufacturer.

Protein extraction and Western blot analysis. MCF-7 cells were lysed and extracted as previously described (11). For immunoblotting, the following primary antibodies were used: p53 DO-1 (1:300; Santa Cruz Biotechnology), p53-pAb1801 (1:100; Santa Cruz Biotechnology), p63 antibody 4A4

(1:400; Santa Cruz Biotechnology), p73 antibody Ab-2 (ER-15 clone, Oncogene; 1:200), antibody anti–ΔNp73 IMG-313A (Imgenex Corporation; 1:100), antibody anti–cyclin A (1:200; Santa Cruz Biotechnology), antibody anti–cyclin D1 (1:200; Santa Cruz Biotechnology), antibody anti–cyclin E (1:200; Santa Cruz Biotechnology), anti-actin Ab-1 antibodies kit (Calbiochem; 1:2,000).

RNA extraction, reverse transcription, and real-time PCR analysis. Total RNA from MCF-7–treated and untreated cells was extracted by using the RNeasy plus mini kit (Qiagen). Reverse transcription reactions were carried out by using the reverse transcription Archive Kit (Applied Biosystems), according to the instructions of the manufacturer. The real-time PCR reactions were performed on an Applied Biosystems 7900HT, as described by the manufacturer. The glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) gene was our internal standard.

Transfections and luciferase assays. Human MCF-7 cells (1×10^5) were cultured 24 h before transfection (~60–80% confluency). Eighty nanomoles per liter of scramble, p53, p63, p73 small interfering RNAs (Dharmacon), 1 μg of pRS vector (control), p53-, p63-, or p73-shRNAs were incubated in 100 μL of serum-free medium and 3 μL of Fugene (Roche) for 20 min at room temperature and subsequently added to the cell cultures.

The fragments containing the p53-responsive elements (RE) of the human *adenosine deaminase* (*ADA*), *fatty acid synthase* (*FASN*), *cyclin D3* (*CCND3*), *regulatory subunit 2 of DNA polymerase δ* (*POLD2*), *tyrosine/threonine phosphatase cell division cycle 25C* (*CDC25C*), and *cell division cycle 2* (*CDC2*) genes were amplified from the human genomic DNA and cloned in the pGL-3 Promoter plasmid (Promega). Transient reporter assays were performed as previously described (12).

For the luciferase assays during cell cycle, MCF-7 and H1299 cells were transfected with recombinant reporter constructs plus scramble or specific p53, p63, and p73 siRNA (Dharmacon) targeting p53, p63, p73 proteins, and 0.01 μg of pRLSV40 (Promega). After transfection, the cells were synchronized by growing in low serum for 48 h and released by the addition of serum for 18 and 22 h. Cells were lysed and luciferase expression was determined by using the Dual-Luciferase Reporter Assay System (Promega).

Chromatin immunoprecipitation assay. MCF-7 cells were cultured in 150 mm culture dishes and synchronized as previously described. At the given time points, proteins were crosslinked to DNA in living nuclei and chromatin immunoprecipitation assay was performed as described (11). The following antibodies were used to immunoprecipitate the DNA-protein complexes: p53 antibody DO-1 (Santa Cruz Biotechnology), p63 antibody H137 (Santa Cruz Biotechnology), p73 antibodies H-79 and C-20 (Santa Cruz Biotechnology), or unrelated control anti-Flag antibody (Sigma).

DNA fragments were analyzed by PCR using specific primers. Primers specific for the coding regions of the different genes or for the unrelated *interleukin-10* promoter were used as negative control.

Results

p63 and p73 proteins are regulated during the cell cycle and their suppression decreases proliferation rate. We first analyzed p53, p73α, and p63α TA and ΔN protein levels in wild-type p53, p63, and p73 MCF-7 cells synchronized by growing in low serum for 48 h and then released by serum restimulation for 14, 18, 22, and 26 h. The cell cycle profile was monitored by flow-cytometric analyses (Fig. 1, *top*) and by immunoblots of cyclins D1, E, and A (Fig. 1, *bottom*), which indicated that MCF-7 cells are arrested in G_1 after 48 h of serum starvation, are at G_1-S transition 14 h after the refeed and in S phase between 18 and 22 h. As shown in Fig. 1, p73α and p63α TA and ΔN protein levels decreased as the cells accumulated in G_0-G_1 (48 h after cell starvation) and increased, albeit with a different timing, after the re-addition of serum. The concentration of TAp73α was higher in S phase cells (18–22 h), whereas ΔNp73α, TAp63α and ΔNp63α accumulation occurred at the G_1-S transition and in early S phase (14–18 h). On

the contrary, p53 and ΔNp53 protein levels were very weakly modulated when the cells moved from G_1 to S phase (14 h; Fig. 1).

These results indicate that, differently from p53 and ΔNp53, the p73α and p63α proteins undergo a coordinated and complex modulation during physiologic cell cycle progression.

In order to assess whether the changes we observed in the p53 family protein levels at the G_1-S transition affect cellular proliferation, we next analyzed the MCF-7 cells subjected to specific siRNA-mediated knockdown of p53, p73, or p63 TA and ΔN isoforms as compared with wild-type cells, by bromodeoxyuridine incorporation and MTT proliferation assays (Fig. 2A and B, *left*). The ability of the different siRNAs to abrogate the expression of p53, p73, and p63 was confirmed by Western blotting (Fig. 2C, *left*) and by
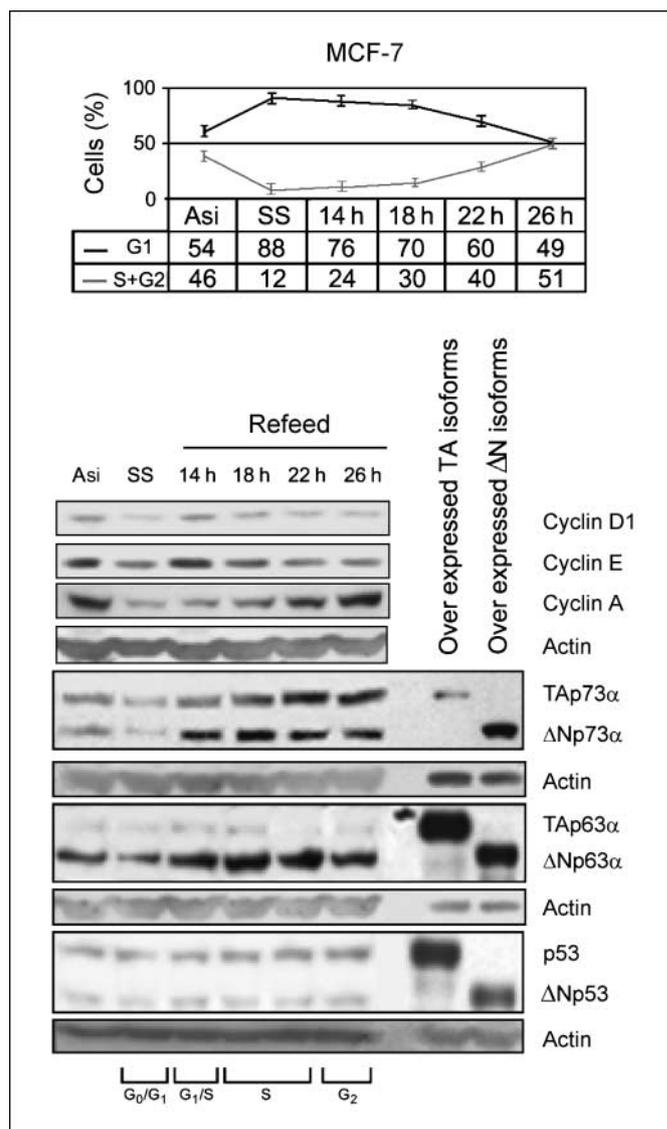
Figure 1. The p63 and p73 proteins are regulated during the cell cycle. MCF-7 cells, which express p53, p63, and p73 proteins, were synchronized by growing in low serum for 48 h (*SS,* serum starvation) and then released by serum addition for 14, 18, 22, and 26 h (refeed). The cell cycle profile was monitored by flow cytometric analysis and immunoblot of cyclins D1, E, and A. Western blot analysis of TAp73α, ΔNp73α, TAp63α, ΔNp63α, and p53 proteins in asynchronous (*Asi*), serum-starved (*SS*), and re-fed MCF-7 cells at the same time points.

Figure 2. p63 and p73 protein suppression in MCF-7 cells reduces cell proliferation. *A,* bromodeoxyuridine (*BrdU*) incorporation after 3-h pulses by MCF-7 cells untransfected, transfected with indicated siRNAs (*left*), with pcDNA3 control vector, or with the indicated expression vectors (*right*). *B,* cell proliferation was measured by MTT reduction in MCF-7 cells untransfected, transfected for 72 h with the indicated siRNAs (*left*), with pcDNA3 control vector, or with the indicated expression vectors (*right*). *C,* the ability of the different specific siRNAs to selectively abrogate the expression of p63, p73, and p53 (*left*) and the levels of exogenously expressed p53 family proteins (*right*) in bromodeoxyuridine and MTT experiments were confirmed by Western blotting.
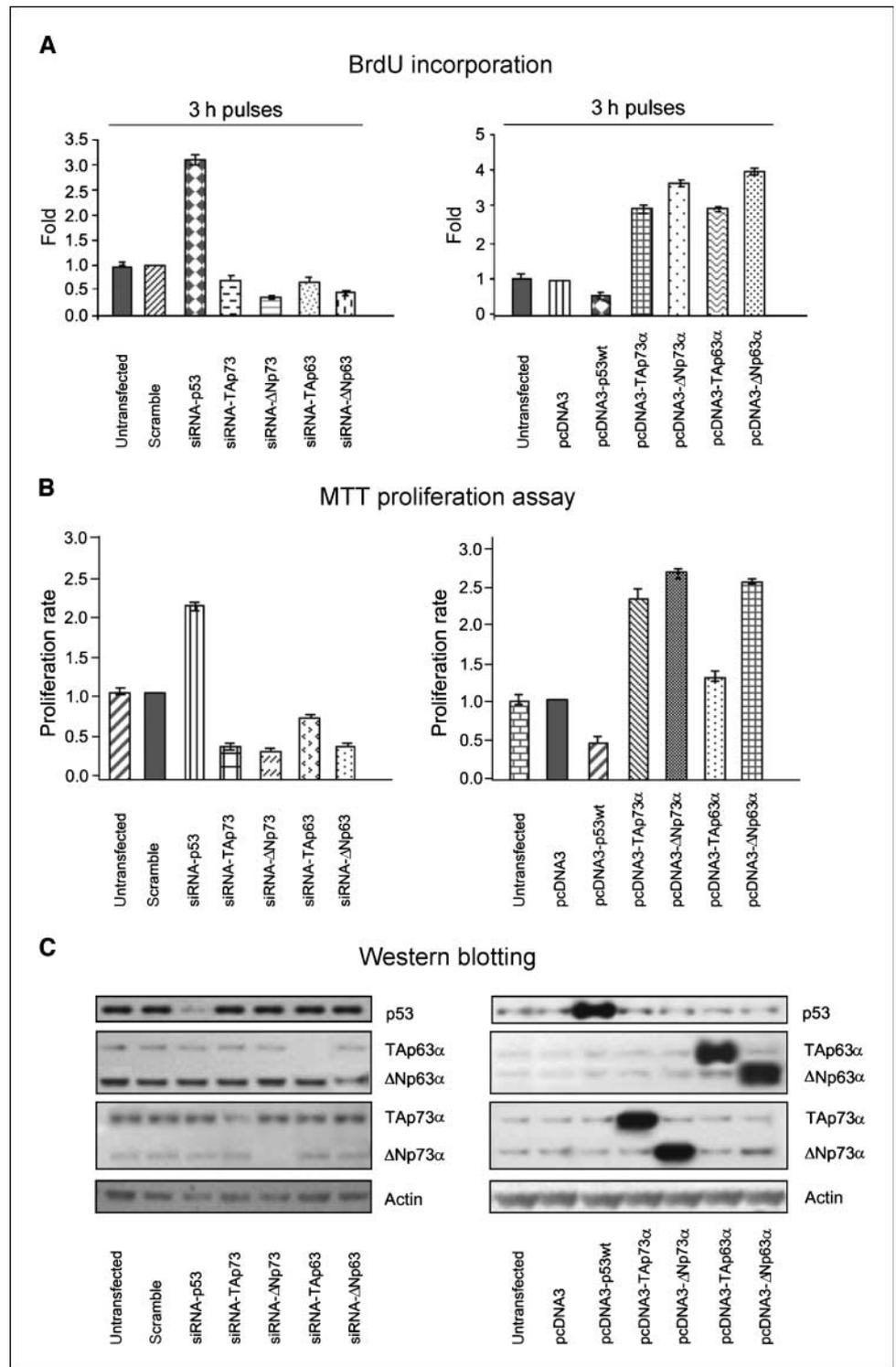
quantitative RT PCR (data not shown). Suppression of p73 or p63 TA and ΔN isoforms led to a reduction in cell proliferation rate as compared both with controls and with the cells that underwent p53 suppression (Fig. 2*A* and *B, left*). The same results were obtained in the MCF-7 cells in which p53, p63, and p73 suppression had been achieved using specific shRNAs, which target the mRNAs in different sequences from siRNAs (Supplementary Fig. S1*A* and *B*). Conversely, the ectopic expression of the p73α and p63α TA

and ΔN isoforms, but not of p53, increased cell proliferation rate (Fig. 2*A, B* and *C, right*).

All these data show that both p63 and p73 TA and ΔN isoforms have effects opposing p53 in the cell cycle under physiologic conditions and that p73 or p63 loss reduced cell proliferation rates.

G_1-S and S phase target genes are differentially regulated by the p53 family members. The reduced proliferation rate of cells lacking p73 and p63 might be explained if these p53 family members

control the expression of the genes involved in growth progression, and specifically, at the G_1-S transition. Indeed, in a computational search across the entire human genome of p53 family REs, we found that many genes involved in G_1-S and G_2-M transitions contain at least one putative p53RE in their regulatory regions (13). We performed a preliminary study, among the *in silico*–selected genes, to choose those genes which were transcriptionally regulated at G_1-S transition because it is well known that many cell cycle genes (e.g., most of the cyclin-dependent kinases) were regulated at the posttranslational level. Therefore, we selected *ADA* (14), *FASN* (12), *CCND3* (15), *POLD2* (16), *CDC25C* (17), and *CDC2* (18) genes for further investigation on the basis of their transcriptional upregulation at the G_1-S transition in serum restimulated MCF-7 cells (Supplementary Fig. S2).

Because p53 and the other p53 family members seem to have opposing effects on cell cycle regulation under physiologic conditions (Figs. 1 and 2*A* and *B*), they should differentially regulate the subset of G_1-S and S phase target genes analyzed. Indeed, we previously showed that *ADA* and *FASN* are direct p73 and p63, but not p53, target genes (11, 12, 14). To test the ability of the different p53 family members to regulate the *ADA, FASN, POLD2, CCND3, CDC25C,* and *CDC2* promoters, we performed transactivation assays. *CDC25C* gene contains two p53REs, Sp1 and NF-Y and CDE (cell cycle–dependent element)/CHR (cell cycle gene homology region) binding sites (Fig. 3, CDC25C). CDE/CHR is a tandem repressor element required for transcriptional repression of a promoter in G_1. The upstream p53RE (that we called p53RE1) overlaps the Sp1 binding site and it has been reported that the binding of p53 to this RE is required for its efficient transcriptional repression (19). Aside from the p53RE1, we identified a second p53RE downstream of the CDE/CHR element that we called p53RE2 (Fig. 3; CDC25C, *left*). The complexity of the *CDC25C* gene regulatory region required four constructs: CDC25C-A containing the p53RE2, CDC25C-B containing the p53RE2 plus the CDE/CHR elements and NF-Y sites, CDC25C-C containing the p53RE1, and CDC25C-D containing all the regulatory sites (Fig. 3, CDC25C, *left*).

We cotransfected the p53-null H1299 cells with the different luciferase reporter constructs containing only the p53REs of each gene considered and recombinant vectors expressing either the human p53 family members (p53, ΔNp53, TAp73α, ΔNp73α, TAp63α, and ΔNp63α; Fig. 3 and Supplementary Fig. S3) or their DNA binding–deficient mutants (p53R175H, TAp73αV156A, TAp63αR279Q, and ΔNp63αR279Q; Supplementary Fig. S4). We found that, although with different efficiencies, both TAp73α and ΔNp73α activated the *ADA, FASN, POLD2, CCND3,* and *CDC2* p53REs and all the constructs of the *CDC25C* regulatory region, whereas p53 and ΔNp53 had no significant activity (Fig. 3; Supplementary Fig. S3). Interestingly, all the p53REs were also activated by ΔNp63α and with less efficiency by TAp63α with the exception of the *ADA* target gene (Fig. 3). As expected, none of the DNA-binding mutants displayed any transcriptional activity (Supplementary Fig. S4). Conversely, the *p21* promoter was activated by p53, TAp73α, and TAp63α.

Altogether, these results indicate that the p53REs identified in *ADA, FASN, CCND3, POLD2, CDC2,* and *CDC25C* genes are differentially targeted by p73, p63, and p53.

The p53REs present in G_1-S and S phase target genes are regulated by endogenous p73, p63, and p53 during cell cycle progression. We also found that the regulation of *ADA, FASN, CCND3, POLD2, CDC25C,* and *CDC2* p53REs by the endogenous

p73 and p63 proteins was modulated during the cell cycle both in p53 wild-type MCF-7 cells (Fig. 4) and in the p53-null H1299 cells (Supplementary Fig. S5). Cells were cotransfected with the indicated luciferase reporter constructs together with scramble (*black columns*) or p53 (*white columns*), p63 (*hatched columns*), and p73 (*light gray columns*) specific siRNAs (Fig. 4; Supplementary Fig. S5). For CDC25C, we transfected both construct A, which contains only the p53RE2 we identified, and construct D, which contains the two p53REs plus the CDE/CHR elements and NF-Y sites (Fig. 4; CDC25C construct A and CDC25C construct D). After transfection, the cells were synchronized in G_0-G_1 and released with serum for 18 to 22 h. The p63, p73, and p53 protein levels and the ability of the different specific siRNAs to abrogate their expression were confirmed by immunoblotting (Supplementary Fig. S6*A*). As cells moved from the G_0-G_1 state to S phase, as confirmed by flow cytometric analyses (data not shown), by the control promoter p21 (Fig. 4, p21) and by cyclin E immunoblot (Supplementary Fig. S6*B*), we observed a significant increase in the transcriptional activity of all the promoters (compare *black columns* in Fig. 4 and Supplementary Fig. S5). Construct D of the *CDC25C* gene showed a higher luciferase expression than construct A in comparing the cells blocked in G_0-G_1 with the cells in early S phase (3.4-fold and 1.8-fold, respectively; Fig. 4), which is due to a synergetic effect of the NF-Y and Sp1 sites with the two p53REs. It is noteworthy that construct A, which lacks the NF-Y and SP1 sites, but not the p53RE2, is still able to induce the luciferase expression 18 h after the refeed. The activation observed in S phase is dependent, for all the p53REs examined, on the endogenous p63 and p73 proteins, but not on endogenous p53, because it is selectively affected by p63 (*hatched columns* versus *black columns*, S phase) or p73 (*light gray* versus *black columns*; S phase) specific siRNAs but not by p53siRNAs (white versus *black columns*, S phase). Because similar results were obtained both in wild-type p53 MCF-7 and in the p53-null H1299 cells, the upregulation of the G_1-S subset of target genes seems to be a direct consequence of p63 and p73 transcriptional activity and to be independent of p53. Interestingly, the abrogation of p53 expression in serum starved MCF-7 cells led to a significant increase in the transcription of all the target promoters with the exception of *CDC2* where the effect was limited (*white columns* versus *black columns*; G_0-G_1), thus suggesting that p53 has a rather repressive role on the transcription of these genes in arrested cells (Fig. 4). p63siRNAs did not modulate significantly the activity of the target promoters either in MCF-7 or H1299 cells in G_0-G_1 (Fig. 4; Supplementary Fig. S5). In agreement with the very low levels of TAp73 proteins in G_0-G_1 MCF-7 (Fig. 1) and H1299 (data not shown) arrested cells, p73 specific siRNAs did not exert a noticeable effect, indicating that p73 is not involved in the regulation of these promoters in quiescent cells.

Finally, in cells transfected with the recombinant reporter vector containing the *p21* promoter (Fig. 4), as expected, the expression of the reporter gene was high in starved cells principally due to p53 activity, because the use of siRNAp53 reduced the luciferase expression compared with controls (compare *white columns* to *black columns* in Fig. 4, *p21*). Upon the addition of serum, when cells enter S phase, there is a marked decrease in luciferase expression of the p21 promoter (compare *black columns* for each time point in Fig. 4, *p21*).

Altogether these data show that when the cells are arrested in G_0-G_1, p53 exerts a significant repressive role on the expression of genes involved in growth progression, whereas p73 and p63 do not have any significant role. In contrast, when the cells move from the

Figure 3. Luciferase assays. The schematic maps of the human genomic regions containing the putative p53REs in *ADA, FASN, CCND3, POLD2, CDC25C,* and *CDC2* genes. The decamers of the REs are boxed and the nucleotides mismatched from the consensus sequences for p53 binding are in lower case. p53-null H1299 cells were cotransfected with wild-type pcDNA3-p53, -ΔNp53, -TAp73α, -ΔNp73α, -TAp63α, and -ΔNp63α expression vectors and the pGL3 promoter-luciferase reporter constructs containing the *ADA, FASN, POLD2, CCND3, CDC25C* construct A, and *CDC2* or *p21* REs. The levels of exogenously expressed p53 family proteins in the H1299 cell line were controlled by Western blot analysis (*bottom*). Cells were lysed and luciferase expression was determined as described. Transfection efficacy was normalized by renilla luciferase activity. *Columns,* average of at least three independent experiments; *bars,* SE.

Figure 4. The p53REs present in *ADA, FASN, POLD2, CCND3, CDC25C,* and *CDC2* target genes are regulated by endogenous p73, p63, and p53 during cell cycle progression. MCF-7 cells were transfected with the pGL3 promoter-*ADA*, pGL3 promoter-*FASN*, pGL3 promoter-*CCND3*, pGL3 promoter-*POLD2*, pGL3 promoter-*CDC25* construct A, pGL3 promoter-*CDC25* construct D, pGL3 promoter-*CDC2*, or pGL3 promoter-*p21* together with scramble RNA (*black columns*), specific siRNAp53 (*white columns*), siRNAp63 (*hatched columns*), or siRNAp73 (*light gray columns*). After transfection, the cells were synchronized in G0-G1 and released with serum for 18 to 22 h to allow the cells to enter into S phase. Similar results were obtained in an independent set of experiments using specific shRNAs targeting p53, p63, and p73 (data not shown). Cells were lysed and luciferase expression was determined as described. Transfection efficacy was normalized by renilla luciferase activity. *Columns,* average of at least three independent experiments; *bars,* SE.
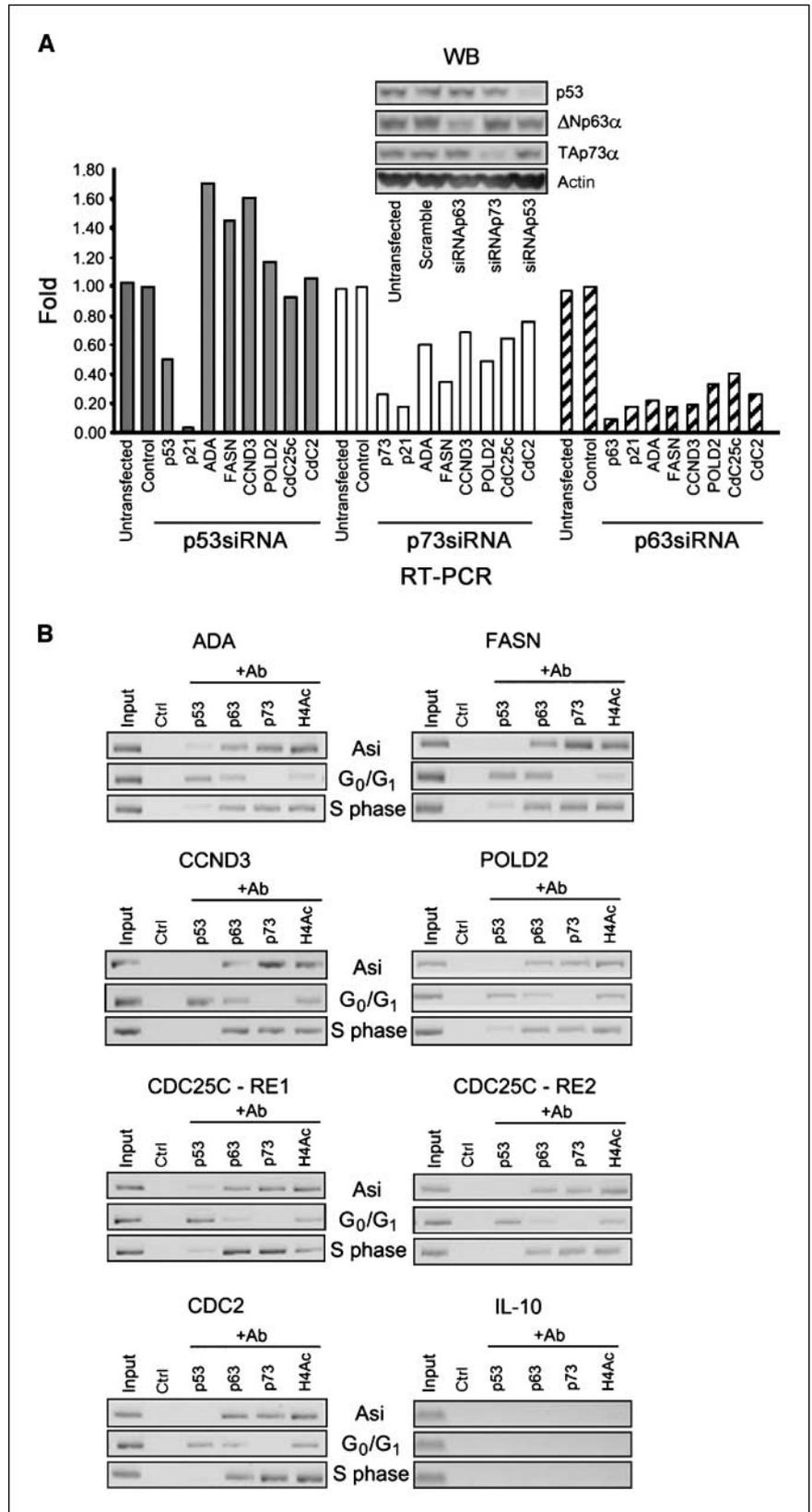
Figure 5. *A,* suppression of p73 or p63 expression reduces the expression of endogenous *ADA, FASN, CCND3, POLD2, CDC25C,* and *CDC2* genes. MCF-7 cells were untransfected or transfected with scramble, siRNA p53, siRNA p73, or siRNA p63. After 24 h, the protein levels of p53, p63, and p73 were evaluated by Western blotting (*top*). The relative quantification of p53, p73, p63, p21, ADA, FASN, CCND3, POLD2, CDC25C, and CDC2 mRNA levels were evaluated by real-time RT-PCR (*bottom*). *B, in vivo* recruitment of p53, p63, p73, and acetylated H4 histone to p53 REs in living cells present in *ADA, FASN, CCND3, POLD2, CDC25C,* and *CDC2* genes during the cell cycle. Crosslinked chromatin was extracted from asynchronous, serum-starved, and re-fed MCF-7 cells, and immunoprecipitated with the indicated antibodies. The immunoprecipitated material was amplified using primers specific for *ADA*-RE or *FASN*-RE, or *CCND3*-RE or *POLD2*-RE, or *CDC25C*-RE1 or *CDC25C*-RE2, or *CDC2*-RE promoters. Primers specific for the coding regions of the different genes or for the unrelated *interleukin-10* promoter were used as negative controls.

G_1 to S phase, p73 and p63, but not p53, induce the expression of these genes.

Transcriptional regulation of endogenous G_1-S and S phase target genes by p73 and p63. Next, we assessed the relative con-

tribution of p73 and p63 proteins in the transcriptional regulation of the *ADA, FASN, POLD2, CCND3, CDC25C,* and *CDC2* endogenous target genes. To this aim, the expression of p53, p63, and p73 was selectively suppressed by transfecting specific p53siRNA, p73siRNA,

or p63siRNA in MCF-7 cells. As shown in Fig. 5A, Western blot and quantitative RT-PCR analyses showed that the cells transfected with each siRNA displayed a decrease in the levels of each mRNA and its corresponding protein. We found that suppression of p53 expression led to an increase in *ADA, CCND3,* and *FASN* expression (*light gray columns* in Fig. 5A) whereas no change in the expression of *POLD2, CDC2,* and *CDC25C* genes was observed, indicating that p53 does not exert any influence or rather has an inhibitory effect on the expression of these genes. A marked decrease was observed in all target mRNAs when cells were transfected with p73siRNAs (*white columns* in Fig. 5A) and, even more strikingly, with p63siRNAs (*hatched columns* in Fig. 5A) whereas the *p21* control target gene was uniformly affected by p53, p73 and p63 specific siRNAs (Fig. 5A). Moreover, in MCF-7 cells, we verified the effects of the transient overexpression of p53, ΔNp53, TAp73α, ΔNp73α, TAp63α, and ΔNp63α on the endogenous levels of all the target genes considered, and we found that their mRNA levels increased following the ectopic expression of the p73α and p63α TA and ΔN isoforms, but not p53 and ΔNp53 overexpression (data not shown).

Therefore, these results support the data reported above and indicate that in asynchronous MCF-7 cells, the depletion of p63 and p73 determines a marked decrease in the expression of the genes implicated in the G_1-S and G_2-M transitions of cell cycle, whereas the ectopic expression of the p73α and p63α TA and ΔN isoforms leads to an increase in their mRNA levels. On the contrary p53 does not exert any control on the expression of *ADA, FASN, CCND3, POLD2, CDC2* and *CDC25C* genes.

p53 family proteins are differentially recruited on the REs of G_1-S and S phase target genes during cell cycle progression. Finally, we sought to correlate the transcription of *ADA, FASN, POLD2, CCND3, CDC25C,* and *CDC2* genes in arrested and serum-released MCF-7 cells with the *in vivo* recruitment of p73, p63, and p53 on their REs using a chromatin immunoprecipitation assay (Fig. 5B). Cross-linked chromatin from asynchronous, growth-arrested, and serum-stimulated MCF-7 cells was immunoprecipitated either with an anti-AcH4 histone or with specific anti-p53, p63, and p73 antibodies. In asynchronously growing cells (*Asi*), p73 and p63, but not p53, were consistently recruited on the REs of all the genes considered (Fig. 5B). In G_0-G_1-arrested cells, the release of p73 and the recruitment of p53 on the same REs were paralleled by a decrease in histone H4 acetylation (Fig. 5B) and in gene transcription (Supplementary Fig. S2, *left*). These results further support the repressive role of p53 on this subset of target promoters in growth-arrested cells. Subsequently, as the cells entered S phase p73 was recruited again on all the promoters whereas p53 binding was sharply decreased (Fig. 5B). p63 binding to the target REs was not significantly modulated in starved cells as compared with asynchronously growing cells, with the exception of the *CDC25C* REs (Fig. 5B), where p63 was completely absent. However, we observed an increase in p63 recruitment on all the promoters in S phase cells. The increased p73 and p63 occupancy was accompanied by both an increase in histone H4 acetylation (Fig. 5B) and in mRNA levels (Supplementary Fig. S2, *right*). Neither the coding regions of our subset of genes nor the *interleukin-10* promoter, which do not contain any p53RE, resulted amplified in the same samples (Supplementary Fig. S7; Fig. 5B, *IL-10*).

Interestingly, neither co-occupation of p53 and p73 nor co-occupation of the three proteins is observed on all the analyzed REs. This means that p53 and p73 independently bind the same RE whereas p63 shares the same RE with p53 or, alternatively, with p73, depending on the conditions.

Discussion

Proper regulation of the cellular division is crucial for the growth and development of all organisms. Understanding the regulation of this process is extremely important for the understanding of many diseases, most notably cancer.

Here, we show in a comprehensive study that p53 family members exercise differential transcriptional control on genes involved in the cell cycle, suggesting a new significant role for p73 and p63 during physiologic cell cycle progression.

Our results clearly indicate that p73 and p63 directly contribute to cell proliferation through the transcriptional activation of a specific subset of target genes. However, this does not exclude that additional cell cycle progression genes are directly regulated by p73 and p63 or the possibility that indirect mechanisms, such as the upregulation of activator protein-1 transcriptional activity (20), might be involved.

Two genes considered in this study, *CDC25C* and *CDC2,* were known to be negatively regulated by the p53 family proteins (21). In particular for *CDC25C* gene, the p53-mediated inhibition involves the direct binding of p53 to the p53RE1 that is overlapped on the Sp1 site (22). It is noteworthy that in our experiments, we found that this p53RE1 (Supplementary Fig. S3 CDC25C-construct C) is downregulated by p53 as reported in literature, but it is activated by p73α TA and ΔN isoforms and by ΔNp63α. In addition, we analyzed the entire regulatory region (CDC25C-construct D) and we found that this construct is again downregulated by p53, but it is activated by p73α TA and ΔN isoforms and by ΔNp63α. Moreover, chromatin immunoprecipitation assays show the *in vivo* binding of p73 and p63 to both *CDC25C* REs in living cells during the transition from G_0-G_1 to S phase (Fig. 5B).

These results also open new possibilities for understanding the involvement of p73 and p63 in cancer. Mutant p53 or elevated concentrations of p73 and p63 proteins could cause aberrant activation of cell growth progression genes in tumors. In fact, p53 is the most frequently mutated gene in cancer and loss of p53 activity is considered to be ubiquitous in all cancers. On the contrary, p63 and p73 genes are rarely mutated and the most common alteration found in human cancer is their overexpression rather than a loss of expression (23). These findings suggest that p73 and p63 play a role in tumorigenesis and it is possible that modulation of p73 and p63 expression contribute to cancer initiation or progression under certain conditions.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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