p53 Regulates Myogenesis by Triggering the Differentiation Activity of pRb

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Abstract. The p53 oncosuppressor protein regulates cell cycle checkpoints and apoptosis, but increasing evidence also indicates its involvement in differentiation and development. We had previously demonstrated that in the presence of differentiation-promoting stimuli, p53-defective myoblasts exit from the cell cycle but do not differentiate into myocytes and myotubes. To identify the pathways through which p53 contributes to skeletal muscle differentiation, we have analyzed the expression of a series of genes regulated during myogenesis in parental and dominant–negative p53 (dnp53)-expressing C2C12 myoblasts. We found that in dnp53-expressing C2C12 cells, as well as in $p53^{-/-}$ primary myoblasts, pRb is hypophosphorylated and proliferation stops. However, these cells do not upregulate pRb and have reduced MyoD activity. The transduction of exogenous *TP53* or *Rb* genes in p53-defective myoblasts rescues MyoD activity and differentiation potential. Additionally, in vivo studies on the *Rb* promoter demonstrate that p53 regulates the *Rb* gene expression at transcriptional level through a p53-binding site. Therefore, here we show that p53 regulates myoblast differentiation by means of pRb without affecting its cell cycle–related functions.

Key words: p53 • Rb • MyoD • differentiation • muscle

Introduction

The differentiation of skeletal myoblasts is characterized by permanent withdrawal from the cell cycle, biochemical and morphological maturation into mononucleated myocytes, and fusion into multinucleated myotubes. This program depends on the expression and activation of myogenic basic helix-loop-helix (bHLH) regulatory factors belonging to the MyoD family (for review, see Weintraub et al., 1991) and on their cooperation with the myocyte-specific enhancer binding factors MEF2 (Melkentin and Olson, 1996; Yun and Wold, 1996). The activity of myogenic bHLH factors is regulated by the interaction with non–tissue-specific proteins. Members of the E2 family were shown to be necessary for binding of the myogenic bHLH factors to DNA, while this binding is inhibited by the presence of "inhibitor of differentiation" factors (for review, see Wright, 1992). Other non–tissue-specific proteins contribute to the cell cycle withdrawal, as well as to the transcription of differentiation-associated genes. These proteins include members of the cyclin-dependent kinase inhibitors, such as $p21^{Waf1/Cip1}$, and cell-cycle regulators, such as pRb. $p21^{Waf1/Cip1}$ is thought to inhibit pRb phosphorylation and thus to promote the cell cycle withdrawal that precedes terminal differentiation. This activity of $p21^{Waf1/Cip1}$ was shown to be p53-independent (Halevy et al., 1995; Parker et al., 1995). The product of the *Rb* gene possesses two different, genetically dissociated functions in the differentiation process (Sellers et al., 1998). The hypophosphorylated form of pRb contributes to the cell-cycle withdrawal by acting as a transcriptional repressor of the E2F-regulated genes, relevant to the cell-cycle progression (Schneider et al., 1994; Weinberg, 1995, and references therein). In addition, pRb cooperates with MyoD to promote the expression of late markers of differentiation (Gu et al., 1993; Chen et al., 1996; Novitch et al., 1996) through the activation of the transcriptionally competent MEF2 factors (Novitch et al., 1999).

The product of the tumor suppressor gene *TP53* is a prominent regulator of cell-cycle checkpoints and apoptotic death. Furthermore, considerable experimental evidence has accumulated suggesting that a fine regulation of the p53 protein activity is required for optimal development and differentiation (for reviews, see Almog and Rot-

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ter, 1997; Choi and Donehower, 1999). p53 expression and/or activity increases during at least some differentiative processes such as hematopoiesis (Kastan et al., 1991), spermatogenesis (Almon et al., 1993), and myogenesis (Halevy et al., 1995; Soddu et al., 1996; Tamir and Bengal, 1998). Exogenous p53 expression can induce differentiation in a variety of tumor cells in vitro (reviewed in Almog and Rotter, 1997) and in vivo (Bossi et al., 2000), while alterations of the *TP53* gene in vivo frequently correlate with undifferentiated phenotypes of cancers (Feinstein et al., 1991; reviewed in Fagin, 1995). By in situ hybridization, it was observed that *TP53* mRNA is present at high levels in all mouse embryonic cells from embryonic day (E) 8.5 to E 10.5. At later stages of development, *TP53* expression becomes more pronounced during the differentiation of specific tissues and declines in mature tissues (Schmid et al., 1991). By using immortal and primary myoblasts and myeloblasts, we have previously demonstrated that the expression of dnp53 proteins strongly reduces terminal differentiation into myotubes and granulocytes/monocytes. In contrast, dnp53 expression does not affect the cell cycle withdrawal and the apoptotic death associated with these differentiation processes (Soddu et al., 1996; Mazzaro et al., 1999). Comparable results were obtained with MyoD-converted $p53^{-/-}$ fibroblasts (Cerone et al., 2000). Interference with p53 mRNA or protein was shown to inhibit the development of *Xenopus laevis* (Wallingford et al., 1997). Despite the normal viability of the original *TP53* null mice (Donehower et al., 1992), it has become increasingly evident that a fine balance of p53 expression and activation is required for the normal development of total embryo or single organs (for review, see Choi and Donehower, 1999). The complete absence of p53 can result in reduced fertility and exencephaly in some mouse strains (Armstrong et al., 1995; Sah et al., 1995), or in mice receiving low folic acid concentration in the diet (Choi and Donehower, 1999). Not less deleterious is the overexpression or the aberrant activation of p53. This was shown by the early embryonic lethality of *TP53* transgenic or *mdm2* null mice, as well as by the rescue of the latter phenotype through the double knock out of the *mdm2* and *TP53* genes (Montes de Oca Luna et al., 1995). All these observations strongly support the involvement of the *TP53* gene in cell differentiation and development. Nevertheless, the pathways modulated by p53 in these processes are still unknown, as are the effectors of these biological events.

To identify the molecules that are downstream of p53 in differentiation, we took advantage of the C2C12 myoblast model we previously employed to demonstrate that dnp53 expression interferes with terminal differentiation into skeletal myotubes (Soddu et al., 1996). Moreover, critical experiments were confirmed on $p53^{+/+}$ and $p53^{-/-}$ primary myoblasts. We compared the expression of myogenesis-regulated genes in parental and dnp53-expressing C2C12 myoblasts, in differentiation promoting conditions. The serial analysis of gene expression and the consequent biochemical and functional studies showed that p53 regulates, at the transcriptional level, the increased expression of pRb, which is involved in the execution of the muscle differentiation program together with MyoD. No modification was found in the pRb-dependent cell-cycle withdrawal, indicating that during myogenesis p53 specifically acts on the differentiation functions of pRb.

Materials and Methods

Differentiation Assay

Mouse satellite cells (MSC)[1] were explanted from new-born mice and maintained in culture as described (Mazzaro et al., 1999). To avoid spontaneous differentiation, primary satellite and C2C12 cells were never allowed to reach confluence. Muscle differentiation was induced by serum withdrawal (DMEM plus 10 μg/ml insulin and 5 μg/ml transferrin, or plus 2% horse serum), as described (Soddu et al., 1996; Mazzaro et al., 1999). Differentiation was evaluated by indirect immunofluorescence for myosin heavy chain (MyHC) (Crescenzi et al., 1994). Differentiation index was calculated by counting at least 400 nuclei per dish, and applying the following formula: differentiation index = number of nuclei in differentiated cells/total number of nuclei.

Northern Blot Hybridization

Total cellular RNA was extracted by RNeasy Mini Kit (QIAGEN), following the manufacturer's instructions. Aliquots of 20 μg per lane were electrophoresed through 1.5% agarose gel in the presence of formaldehyde. Gels were blotted onto Duralose nitrocellulose membrane (Stratagene) and hybridized according to standard protocols (Ausbel et al., 1987). Probes were gel-purified and labeled with [^{32}P]-dCTP (Dupont) by random primer extension (Ausbel et al., 1987).

Western Blotting

Cells were plated in 60-mm dishes (7.5×10^4/dish) and incubated in growth medium (GM) or in differentiation-promoting medium (DM) for different times. Approximately 30 min before harvesting the cells, Hoechst 33258 dye was added to the culture medium (GM or DM) to a final concentration of 1 μg/ml, to stain nuclei. 20 different microscopic fields for each sample were analyzed by fluorescent microscope to estimate the total number of nuclei per dish. Equal numbers of nuclei were lysed in hot Laemmli buffer, loaded on 7 or 10% SDS-polyacrylamide gels, electrophoresed, and blotted onto nitrocellulose membranes (Bio-Rad Laboratories). Filters were immunoreacted with anti–pRb G3-245 mAb (PharMingen), anti–MyHC MF-20 mAb (Bader et al., 1982), or anti–Hsp70 N27F3-4 mAb (StressGen Biotechnologies). Immunoreactivity was determined using the ECL-chemiluminescence reaction (Amersham Corp.) following the manufacturer's instructions.

Plasmids, Viruses, and Cell Transduction

The following plasmids were used for transfections: pN53cG(Val135), carrying the temperature-sensitive $p53Val^{135}$ mutant gene (Michalovitz et al., 1990) and the neo gene; pRSVneo, carrying the selectable marker for G418 resistance; pLxSP, carrying the selectable marker for puromycin resistance; pMCK-luc, carrying 1,256 base pairs of the muscle creatine kinase (MCK) promoter upstream of the luciferase reporter gene (Novitch et al., 1999); pXRP1 and pd92, carrying the luciferase reporter gene driven by the human Rb promoter between −686 and −4 and between −686 and −92, respectively, relative to the translation start site (Osifchin et al., 1994); and pXRP1-mtp53, obtained from the pXRP1 vector by the QuikChange site-directed mutagenesis kit (Stratagene). In the last vector, the sequence of the p53 binding site (between −82 and −73) 5′-GGGCGTGCCC-3′ was mutated in 5′-TGTCGTTAAC-3′, and the mutation was confirmed by direct sequencing. C2C12 cells were transfected by $CaPO_4$-mediated DNA precipitation by using a 1:20 molar ratio between resistance and experimental vectors, when required. Selection was performed in the presence of 0.75 mg/ml G418, or 2 μg/ml puromycin. dnp53 protein expression was assessed by indirect immunofluorescence and Western blotting (Soddu et al., 1996). Integration of luciferase reporter vectors was assessed by direct PCR using two primers complementary to the luciferase gene.

[1]*Abbreviations used in this paper:* Act D, actinomycin D; DM, differentiation-promoting medium; dnp53, dominant-negative p53; GM, growth medium; MCK, muscle creatine kinase; MSC, mouse satellite cell; MyHC, myosin heavy chain.

Recombinant adenoviruses Adp53 (Bacchetti and Graham, 1993), encoding a human wt-p53 protein and Ad-ΔRb (Chang et al., 1995), encoding a low phosphorylable human Rb protein, and the replication defective, E1-deleted dl312 control adenovirus were prepared and titrated by 293-cell infection. Recombinant retroviruses Ltsp53SN, encoding p53Val^{135} mutant and the insertless LxSN, were produced by GP+E packaging cells.

Luciferase Activity

Luciferase activity was assessed as described (Osifchin et al., 1994) and normalized as reported for Western blotting.

In Vivo Footprinting and Chromatin Immunoprecipitation

For in vivo footprinting, proliferating and differentiated cells were treated with 0.06% dimethyl sulfate (Sigma-Aldrich) for 30 s. DNA was extracted and cleaved with piperidine (Sigma-Aldrich). As control, purified genomic DNA was methylated in vitro with 0.125% dimethyl sulfate for 2 min. In vivo footprintings were performed by legation-mediated PCR (Martinez-Balbas et al., 1995) using the following primers: 5′-CCCGACTCCCGTTACAAAAATAATG-3′ (56.33°C), 5′-GAACGTCCCCCGAGGAAAACCG-3′ (61.65°C), and 5′-AACGTCCCCCGAGGAAAACCGGACGC-3′ (65.8°C).

For chromatin cross-linked immunoprecipitation, proliferating and differentiated cells were cross-linked by adding formaldehyde directly to culture medium. DNA preparation, immunoprecipitation, and amplification were performed as described (Boyd et al., 1998). Affinity purified rabbit or sheep polyclonal antibodies were employed: anti–Sp1 sc-59-X (Santa Cruz Biotechnology, Inc.) and anti–p53 Ab-7 (Calbiochem). The following primers were employed to amplify the indicated promoters: the murine *Rb* promoter, 5′-GACGACGCGGGCGGAGACAGG-3′, 5′-AACGTCCCCCGAGGAAAACCGGACGC-3′; the human *Rb* promoter upstream the luciferase gene, 5′-GAGGGCGCGTCCGGTTTTTCTCA-3′, 5′-AGCAATTGTTCCAGGAACCAGGGCATAGC-3′; the murine *MyoD* promoter, 5′-CCTGGGGCTATTTATCCCCAGGGTAGCC-3′, 5′-TAGACCACTGGAGAGGCTTGGGCAG-3′; the murine myosine light chain promoter, 5′-CTTCAGTCTCACCAGGGCTGTTCAC-3′, 5′-CTCTCTCTGGCTTCCTTTTTATTTCTGGGC-3′, the murine desmin promoter, 5′-TGCTTCCTAGCTGGGCCTTTCC-3′, 5′-CCGGACAG-GTCTCTACCATCCTTTC-3′.

Results

Myoblasts from p53$^{-/-}$ Mice Have Reduced Differentiation Capacity

We have previously demonstrated that the expression of dnp53 proteins in primary and immortalized myoblasts inhibits their differentiation (Soddu et al., 1996; Mazzaro et al., 1999). These dnp53 proteins should form mixed, transcriptionally inactive oligomers with the endogenous wt-p53 protein (Milner and Medcalf, 1991). Recently, some weak but transcriptionally relevant heterotypic interactions have been described between some mutant p53 and p63 or p73 proteins (Davison et al., 1999; Di Como et al., 1999), which are members of the newly discovered p53 family (for reviews, see Oren, 1997; Kaelin, 1999). Since p63 and p73 knock-out mice present more evident developmental defects than p53$^{-/-}$ mice (Mills et al., 1999; Yang et al., 1999, 2000), it is possible that the inhibition of myoblast differentiation induced by the dnp53 expression is due to the inactivation of other p53 family members. We have shown that MyoD-converted, p53$^{-/-}$ fibroblasts have a significant reduction in their differentiation capacity, compared with the p53$^{+/+}$ fibroblasts (Cerone et al., 2000). Still, to more directly demonstrate a p53-specific role in myogenesis, primary myoblasts were explanted from p53$^{+/+}$ and p53$^{-/-}$ mice to study their differentiation capacity.

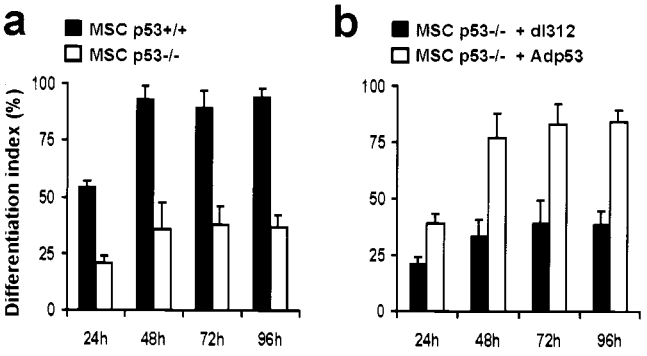

Figure 1. Effects of p53 impairment on muscle differentiation. (a) Time-course analysis of differentiation indices of mouse satellite cells (MSC) explanted from p53$^{+/+}$ and p53$^{-/-}$ mice. (b) p53$^{-/-}$ MSC were induced to express wt-p53 protein by infection with the Adp53 recombinant adenovirus. The E1A-deleted dl312 adenovirus was used as negative control. Time course analysis of differentiation indices was performed as in a.

Time course analyses of differentiation showed a strong and persistent reduction of differentiation indices in p53$^{-/-}$, compared with p53$^{+/+}$ myoblasts (Fig. 1 a). This reduction was completely rescued by the expression of an exogenous wt-p53 protein obtained by myoblast infection with a p53-carrying recombinant adenovirus (Bacchetti and Graham, 1993) (Fig. 1 b). These results demonstrate that the other members of the p53 family cannot substitute the p53-specific role in muscle differentiation.

p53-defective Myoblasts Do Not Upregulate Rb Expression

To identify the molecules downstream of p53 in the differentiation process, we evaluated the expression of a series of muscle differentiation–regulated genes in myoblasts derived from the C2C12 cell line. Total RNA was extracted from parental (C2C12) and dnp53-expressing (C2-dnp53) myoblasts (Soddu et al., 1996) maintained in GM, or incubated in the presence of DM for different times. As expected, C2-dnp53 cells had a strong reduction in the expression of muscle structural genes, like *MyHC*, compared with the parental cells (Fig. 2, a and b). A series of genes, including *MyoD*, *myogenin*, *p21$^{Waf1/Cip1}$*, *cyclin D1* and *D3*, and *Id-1*, were found to behave similarly in the two cell types (Fig. 2 a). In contrast, a strong difference in the *Rb* mRNA induction was present between parental and C2-dnp53 cells (Fig. 2, a and c), the latter being largely impaired in their ability to increase the *Rb* mRNA levels. This difference was even more marked at the protein level (Fig. 2 d) and was confirmed in p53$^{-/-}$ primary myoblasts (e). Moreover, infection of the p53$^{-/-}$ primary myoblasts with recombinant retroviruses carrying the dnp53 protein did not further inhibit MyHC and Rb expression (Fig. 2 e) and differentiation indices (15.3 and 16.7% in neo and dnp53-expressing cells, respectively), supporting the p53 specificity for this differentiation activity. Interestingly, Rb expression in proliferating conditions was similar in parental and p53-impaired cells, suggesting a specific p53 control on the differentiation-associated upregulation of the *Rb* gene.

To evaluate whether the differentiation-associated *Rb* upregulation was stably inhibited in the C2-dnp53 cells, or

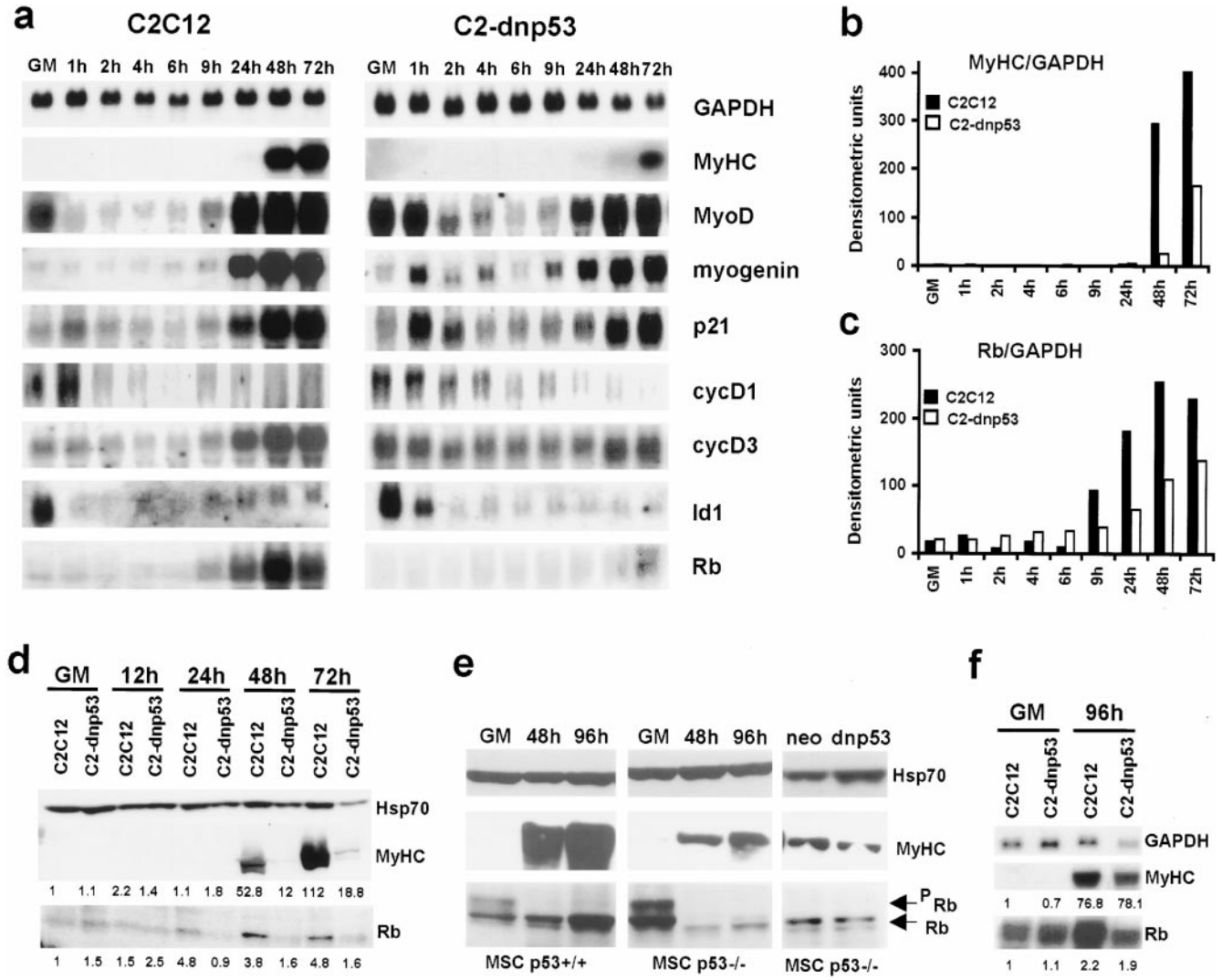

Figure 2. Serial analysis of muscle differentiation–regulated gene expression. (a) Northern blot hybridizations were performed on total RNA extracted from C2C12 and C2-dnp53 cells incubated in DM for the indicated times, or maintained in GM. (b and c) Quantitative analyses, relative to GAPDH, of the gene expression levels observed in a were evaluated for *MyHC* and *Rb* in the indicated cells. (d) C2C12 and C2-dnp53 cells, incubated as in a, were analyzed by Western blotting for the listed proteins. The numbers represent quantitative data relative to Hsp70. (e) $p53^{+/+}$ and $p53^{-/-}$ MSC were incubated as in a. $p53^{-/-}$ MSC were also transduced with the indicated gene by retroviral infection and incubated for 96 h in DM. Total cell lysates from each of these cell lines were analyzed by Western blotting for the listed proteins. (f) C2C12 and C2-dnp53 cells were incubated in GM, or in DM for 96 h, and maintained at 32°C. Northern blot hybridization was performed for the indicated genes. The numbers represent quantitative data relative to GAPDH.

this inhibition was dependent on the sustained expression of exogenous dnp53 protein, we took advantage of this particular dnp53 protein being temperature sensitive (Michalovitz et al., 1990). Northern blot analysis was performed on parental and C2-dnp53 cells at the permissive temperature (32°C). The complete recovery of the differentiation ability of C2-dnp53 cells at 32°C (Soddu et al., 1996) was associated with that of *MyHC* and *Rb* expression levels (Fig. 2 f). These results indicate that inhibitions of differentiation and of *Rb* upregulation are reversible consequences of p53 inactivation.

p53 Does Not Interfere with the Cell Cycle-related Functions of pRb

Skeletal muscle cells belong to those cell types in which differentiation is associated with irreversible withdrawal from the cell cycle (Crescenzi et al., 1995). In differentiating conditions, $Rb^{-/-}$ myoblasts are defective in cell cycle withdrawal and expression of late markers of differentiation (Schneider et al., 1994; Novitch et al., 1996). Furthermore, those $Rb^{-/-}$ myoblasts that stop proliferating and reach differentiation can re-enter the cell cycle after serum stimulation (Schneider et al., 1994). Since C2-dnp53 cells, which stop proliferating upon induction of differentiation (Soddu et al., 1996), are drastically inhibited in their differentiation capacity and do not upregulate *Rb* (as assessed at the population level), we evaluated whether these cells can re-enter the cell cycle upon serum stimulation. We found that most of the MyHC-negative, undifferentiated cells, which stop proliferating after serum starvation, reactivated DNA synthesis as well as parental cells (80% in the C2-dnp53 population and 70% in the parental one). By contrast, MyHC-positive C2-dnp53 cells were not able to

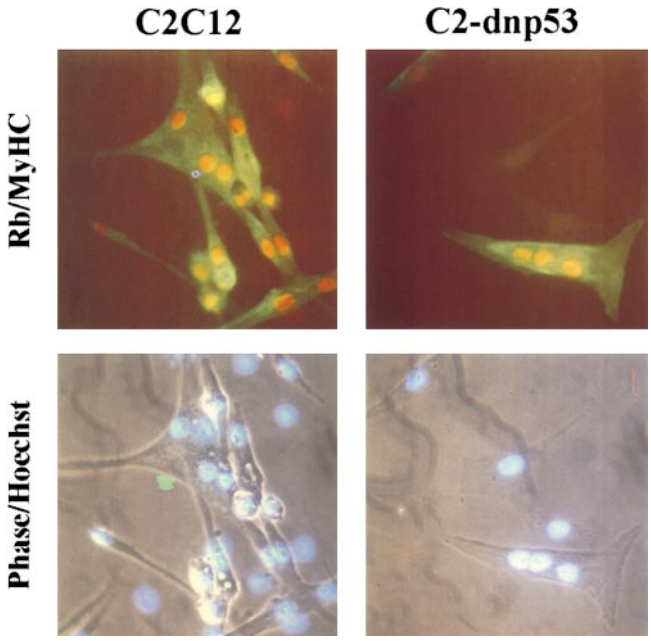

Figure 3. pRb expression in differentiated myotubes. (Top) Indirect immunofluorescence performed on C2C12 and C2-dnp53 cells 72 h after the incubation in DM. Anti–pRB mAb was revealed by TRITC-conjugated antiserum and anti–MyHC serum by FITC-conjugated antiserum. (Bottom) Hoechst-stained nuclei pictures superimposed on the relative phase contrast images of the same fields shown above.

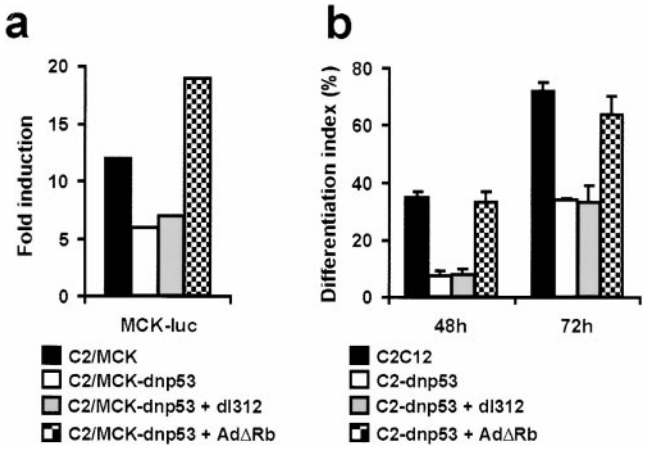

Figure 4. Effects of pRb overexpression in C2-dnp53 cells. (a) Activity of the MCK promoter in the cells indicated. C2C12 cells were stably transfected with the MCK reporter vector and subsequently infected with dnp53-carrying or insertless retrovirus. The C2-MCK-dnp53 cells were also infected with Ad-ΔRb virus to increase pRb expression or dl312 adenovirus as control. Fold induction values indicate the ratio between the values obtained in DM (48 h) and GM conditions. (b) Differentiation indices were evaluated at the indicated time points in C2C12 and C2-dnp53 cells before and after infection with adenovirus as in a.

reactivate the cell cycle any longer. Indeed, we could never find BrdUrd/MyHC double-positive cells in all the experiments performed. In addition, the expression of pRb protein was analyzed at the single-cell level by indirect immunofluorescence. As shown in Fig. 3, the C2-dnp53 cells that could differentiate had increased levels of pRb. Moreover, the hypophosphorylation of pRb, which is responsible for cell cycle withdrawal, was similarly present in parental and dnp53-expressing cells (data not shown), as well as in $p53^{+/+}$ and $p53^{-/-}$ myoblasts (Fig. 2 e). Taken together, these results indicate that p53 is not essential for the cell cycle–related functions of pRb.

p53 Controls the Differentiation Activity of pRb

A pRb function genetically distinct from cell cycle control affects the transcription of differentiation-related genes (Sellers et al., 1998; Novitch et al., 1999), pRb being required for the full function of the transcription factor MyoD during skeletal muscle differentiation (Gu et al., 1993; Novitch et al., 1996, 1999). Thus, MyoD transcriptional activity was measured in C2C12 and C2-dnp53 cells stably transfected with a reporter vector containing the MCK promoter. Upon induction of differentiation, a considerable accumulation of the luciferase reporter was found in parental C2C12 cells, while a strongly reduced accumulation was detectable in C2-dnp53 cells. This reduction was fully suppressed by infection with a recombinant adenovirus encoding an active pRb (Ad-ΔRb; Chang et al., 1995) (Fig. 4 a). Since MyoD activity was rescued by exogenous pRb overexpression, we evaluated the differentiation capacity of these cells. As shown in Fig. 4 b, Ad-ΔRb–infected C2-dnp53 cells differentiate as efficiently as the parental C2C12 cells, indicating that exogenous pRb overexpression is sufficient to recover the differentiation capacity of C2C12 cells lacking p53 activity. Altogether, these results strongly indicate that p53 controls the differentiation function of pRb.

Differentiation-associated Rb Upregulation Is Mediated at the Transcriptional and Posttranscriptional Levels

It has been suggested that, during myoblast differentiation, *Rb* mRNA upregulation is transcriptionally mediated (Martelli et al., 1994; Okuyama et al., 1996). Thus, the luciferase reporter vector pXRP1, which carries the −686 to −4 base-pair fragment of the human *Rb* promoter (Osifchin et al., 1994), was stably transfected into C2C12 cells. The kinetics of luciferase expression was evaluated in two different polyclonal populations upon induction of differentiation and compared with the levels of proliferating cells (time 0). A strong reduction in luciferase activity was present 24 h after differentiation induction. This initial downregulation was followed by the recovery of the promoter activity at the 48 h time point (Fig. 5 a), with no considerable difference between the two populations analyzed (b). Interestingly, in all the time courses performed, the differentiation-associated luciferase activity never reached the level measured in proliferating cells. This suggests the presence of a different type of *Rb* gene transcription between the proliferating and differentiating states. Thus, to detect possible differential occupations of cis-acting elements in the two conditions, in vivo footprintings were performed on the endogenous *Rb* promoter of C2C12 cells. The *Rb* promoter region responsible for the regulation during in vitro myogenesis (Okuyama et al., 1996) was analyzed. Proliferating and 48 h-differentiated cells showed different patterns of nucleotide protection (Fig. 5 c), supporting the idea of the presence of different transcriptional complexes in the two conditions compared.

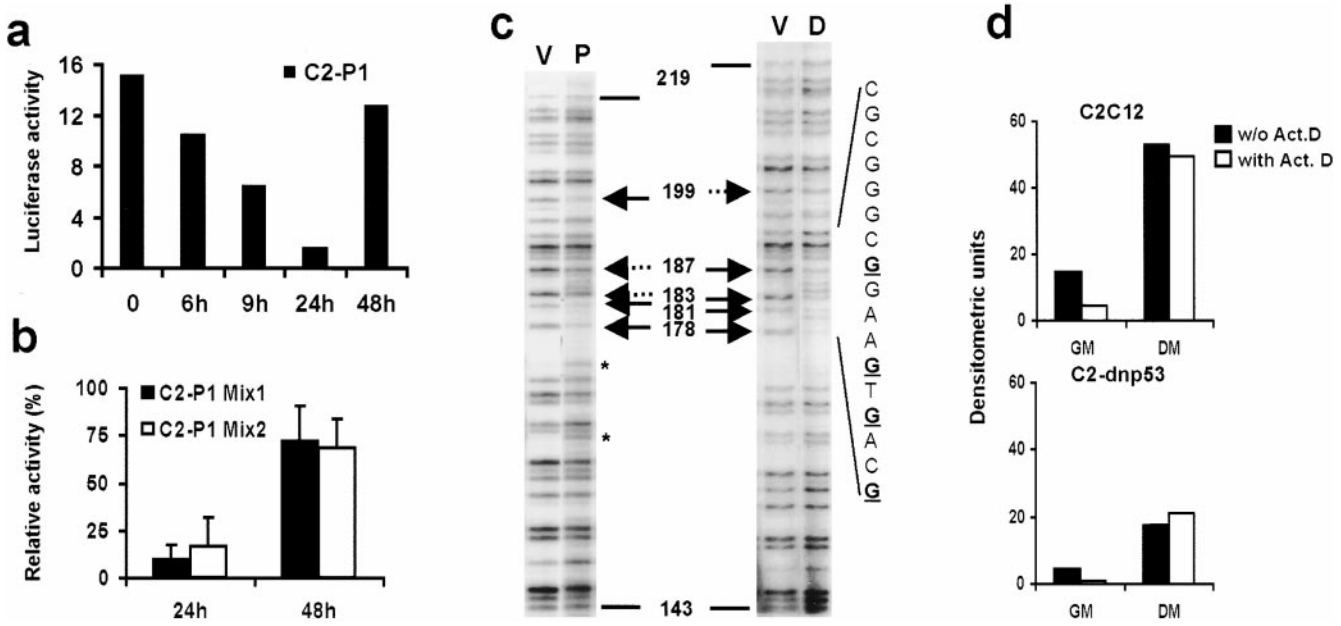

Figure 5. Rb promoter activity in muscle differentiation. (a) pRXP1-carrying C2C12 cells (C2-P1) were incubated in DM for different times. Time 0 indicates cells maintained in GM. Luciferase activity was normalized for number of nuclei. (b) Two different polyclonal populations of pRXP1-carrying C2C12 cells (C2-P1 Mix1 and C2-P1 Mix2) were incubated as in a. Columns report the luciferase activity of each cell line, relative to its own GM. (c) The region between −219 and −143 base-pairs upstream of the translation start site of the C2C12 endogenous *Rb* promoter was analyzed by in vivo footprinting in proliferating (P) and 48 h differentiated (D) cells; in vitro footprinting (V) was used as control. (Continuous arrows) Bands that are significantly protected; (dashed arrows) bands that are not protected; and (*) spurious bands. (d) Total RNA was extracted from C2C12 and C2-dnp53 cells maintained in GM or DM for 48 h, with or without Act D. Northern blottings were performed for the *Rb* and *GAPDH* genes. Histograms show quantitative analyses of the *Rb* gene expression levels relative to *GAPDH*.

Since the increment of *Rb* mRNA levels during C2C12 differentiation is not proportional to the increase in the *Rb* promoter activity in the same conditions, we asked whether posttranscriptional regulation of the *Rb* gene expression was also present. Northern blot analyses were performed on total RNA extracted from C2C12 and C2-dnp53 cells treated with the transcription inhibitor Actinomycin D (Act D). In proliferating conditions, reduction of *Rb* mRNA levels was found in both cell types compared with the relative untreated cells. At 48 h from differentiation induction, comparable amounts of *Rb* mRNA were present in cells treated and untreated with Act D, although, as expected, the absolute levels were different between parental and C2-dnp53 cells (Fig. 5 d).

Altogether, the results reported in this paragraph demonstrate that the differentiation-associated *Rb* upregulation is mediated at the transcriptional and posttranscriptional levels and that the latter type of control is p53 independent.

During Muscle Differentiation, p53 Contributes to the Transcriptional Regulation of the Rb Promoter through a p53-binding Site

Since the p53 transcriptional activity is required for the induction of differentiation (Soddu et al., 1996), we evaluated whether p53 contributes to the *Rb* gene transcription. pXRP1-carrying C2C12 cells were stably transduced with the dnp53 protein by retroviral infection and maintained as polyclonal populations. When these cells were incubated in DM, the *Rb* promoter showed the 24 h silencing that, however, was not followed by the normal 48 h recovery of transcriptional activity (Fig. 6 a). This finding indicates that p53 contributes to the increase of the *Rb* promoter activity that occurs during C2C12 differentiation after the initial silencing. The timing of this recovered activity correlates with the p53-specific transcriptional activity that we previously measured on the artificial p53-reporter vector PG_{13}-CAT (Soddu et al., 1996). Notably, no difference in *Rb* promoter activity was found between parental and dnp53-expressing C2C12 cells in proliferating conditions, as assessed by stable and transient transfections of C2C12 and C2-dnp53 cells with the pXRP1 (data not shown). These results further support the idea that p53 is involved only in the differentiation-associated upregulation of the *Rb* gene.

Since a sequence positively regulated by p53 had been mapped in the *Rb* promoter between −92 and −4 base-pairs upstream of the translation start site (Osifchin et al., 1994), we stably transfected into C2C12 cells a pXRP1 vector carrying a deletion of this region (pd92) (Osifchin et al., 1994). As shown in Fig. 6 b, after the expected promoter silencing at 24 h, no activation of pd92 transcription was present at 48 h, supporting the hypothesis that during C2C12 differentiation p53 modulates the *Rb* promoter through the p53-binding site present in this region. To confirm this hypothesis, the p53-binding site was mutated in the pXRP1 vector, creating the pXRP1-mtp53 that was stably transduced into C2C12 cells. Upon differentiation induction, the levels of luciferase activity of pd92 and pXRP1-mtp53 were comparable (Fig. 6 b), indicating that p53 regulates *Rb* transcription through this p53-binding site.

Because of the particularly high content in Guanine–Cytosine of the *Rb* promoter region containing the p53-binding site, we were not able to perform in vivo footprintings of this site. Thus, to verify the in vivo p53 binding to the

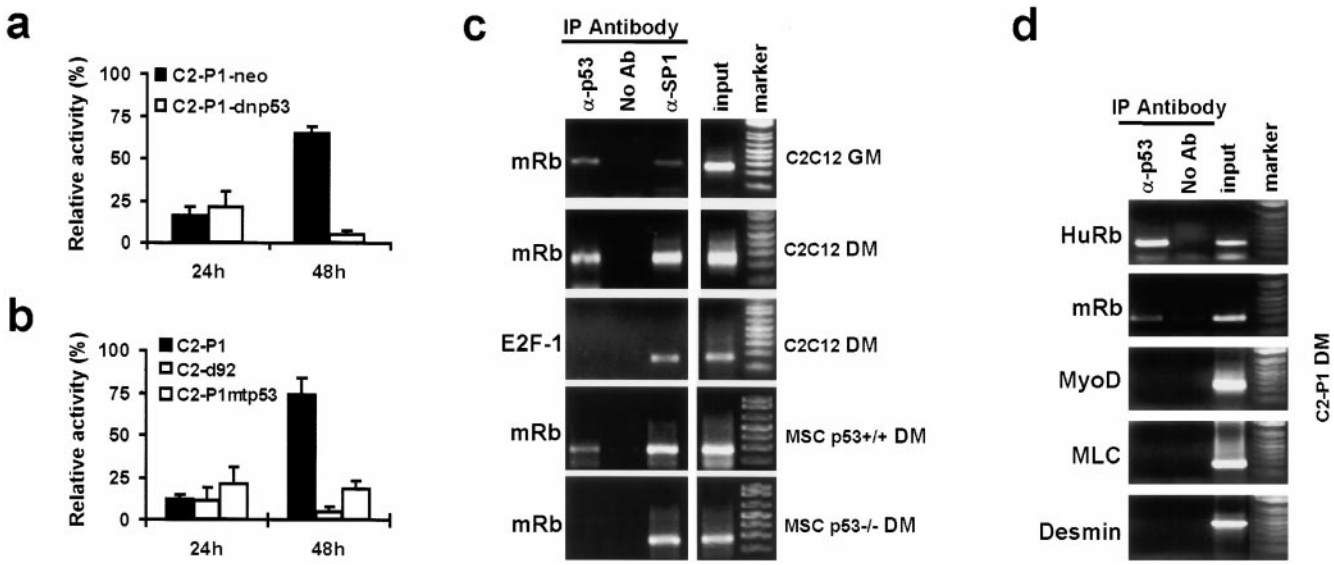

Figure 6. p53 activity on the Rb promoter. (a) C2-P1 cells were infected with a recombinant retrovirus carrying the dnp53 gene or its insertless control. Relative luciferase activity was assessed on polyclonal populations as described in Fig. 5. (b) C2C12 cells were stably transduced with pXRP1 (C2-P1), pd92 (C2-d92), and pXRP1-mtp53 (C2-P1mtp53). Relative luciferase activity was assessed on polyclonal populations as in a. (c and d) Cross-linked chromatin from the indicated cells was immunoprecipitated with antibodies to p53, Sp1, or in the absence of antibodies, and analyzed by PCR with primers specific for the indicated promoters (see Materials and Methods). Sp1 was used as positive control in the promoters with an SP1-binding site; input corresponds to nonimmunoprecipitated cross-linked chromatin.

proximal region of the *Rb* promoter, a chromatin–cross-linked immunoprecipitation was performed (Boyd et al., 1998) in C2C12 cells maintained in the presence of GM or DM and in $p53^{+/+}$ and $p53^{-/-}$ MSC incubated in DM. Proteins were cross-linked to DNA by direct incubation of living cells with formaldehyde. DNA was extracted, immunoprecipitated with anti–p53 or anti–SP1 sera, and the *Rb* promoter sequence was amplified from the immunoprecipitate by PCR. We found the proximal region of the *Rb* promoter in the p53-bound cross-linked chromatin of C2C12 and $p53^{+/+}$ MSC (Fig. 6 c), indicating the presence of p53 in the complexes that bind the *Rb* promoter in vivo. The Rb promoter sequence could not be amplified from the anti–p53 immunoprecipitated chromatin obtained from $p53^{-/-}$ MSC, demonstrating the specificity of the anti–p53 immunoprecipitation. In the same experiment, the E2F promoter that contains the SP1-binding site, but not the p53-binding site, was used as control. To further confirm p53 binding to the Rb promoter, the C2-P1 cells carrying the exogenous human Rb promoter were in vivo cross-linked. The sequence of exogenous human and endogenous murine Rb promoters, as well as a series of muscle specific promoters that do not contain p53-binding sites were amplified from the same DNA immunoprecipitations (Wells et al., 2000). As shown in Fig. 6 d, both human and mouse Rb promoters could be amplified, while no amplification was detectable in the control promoters. These results support the conclusion that p53 binds the Rb promoter in muscle cells.

Discussion

The *TP53* gene has been largely characterized as a cell-cycle checkpoint controller and an apoptosis inducer. In addition, different studies performed in vivo and in vitro have indicated a p53 involvement in embryonic development and in cell differentiation. However, the mechanisms through which p53 influences differentiation and development are substantially unknown. In the present report, we show that, during skeletal muscle differentiation, p53 is required to establish the increased level of pRb expression, which regulates the further steps of muscle differentiation (Fig. 7).

The pRb requirement in skeletal muscle differentiation has been extensively studied. Inactivation of the pRb function by proteins from DNA-tumor viruses, as well as studies performed with $Rb^{-/-}$ cells have shown that pRb is involved in cell-cycle withdrawal and in the expression of late markers of differentiation (Gu et al., 1993; Chen et al., 1996; Novich et al., 1996, 1999). These two pRb functions can be genetically and mechanistically dissociated, as it has been elegantly demonstrated by studying pRb mutants from high- and low-risk retinoblastomas (Sellers et al., 1998). pRb can inhibit DNA synthesis thanks to its ability to bind E2F and DNA, and to repress transcription through a specific domain. In contrast, the abilities to activate transcription and promote differentiation do not require E2F binding, but rather need cooperation with other types of transcription factors, such as MyoD. We found that, in the presence of differentiation-promoting stimuli, p53-impaired myoblasts maintain the capacity to hypophosphorylate pRb and to withdraw from the cell cycle, while losing their capacity to increase pRb expression and have strongly reduced MyoD activity and differentiation potential. These findings indicate that p53 discriminates between the cell cycle– and differentiation-related functions of pRb and provide a biological counterpart to the biochemical characterization of the pRb mutants described above (Sellers et al., 1998). Furthermore, our results are in good agreement with the observations indicating that the pRb hypophosphorylation responsible for the differentiation-associated cell cycle withdrawal involves a p53-independent $p21^{Waf1/Cip1}$ upregulation (Halevy et al., 1995; Parker et al., 1995).

In physiological proliferating conditions, our p53-impaired myoblasts did not show any modification of the *Rb* gene expression, as shown by Northern and Western blot,

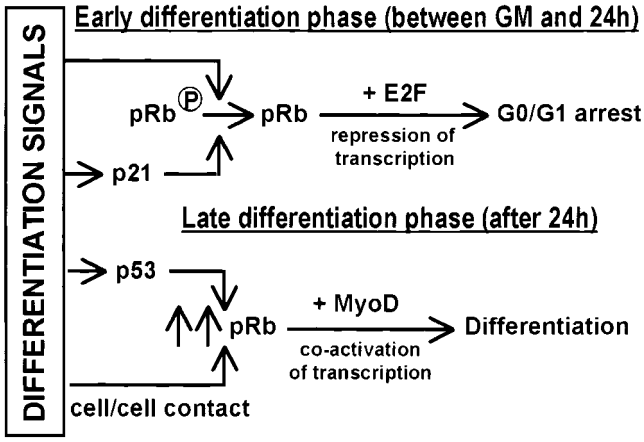

Figure 7. A model to explain p53 role in the induction of skeletal muscle differentiation. Early steps of differentiation include the activation of $p21^{Waf1/Cip1}$ and the hypophosphorylation of pRb, which leads to the transcriptional repression of the E2F-activated genes. Findings (Halevy et al., 1995; Parker et al., 1995; Soddu et al., 1996; this report) indicate that these steps are p53 independent. After the cell-cycle withdrawal, p53 modifies the *Rb* gene transcription and contributes to the increase of pRb expression levels. Therefore, the upregulated pRb can cooperate with MyoD to transcribe later markers of differentiation. Cell-to-cell contacts can also increase pRb expression, overcoming the p53 requirement (Coen, S., and S. Soddu, unpublished results). Further and later functions of p53, such as the direct transcription of MCK promoter (Tamir and Bengal, 1998) are not included in this model since they are related to the activation of structural rather than regulatory genes.

and by promoter activation analyses. These observations are consistent with the notion that p53 is not involved in cell cycle control in normal proliferating conditions. In contrast, it is well known that different types of stressing stimuli promote p53 activation. In this type of situation, p53 is known to promote pRb hypophosphorylation and inhibition of DNA synthesis through the transcriptional induction of $p21^{Waf1/Cip1}$ (Dulic et al., 1994). Indeed, compared with the parental cells, C2-dnp53 cells do not arrest in the G1 phase of the cell cycle in response to doxorubicin-induced DNA damage (Manni et al., manuscript submitted for publication). Together with the findings we obtained in differentiating conditions, these results indicate the presence of two different types of p53-dependent regulation of pRb. One operates through $p21^{Waf1/Cip1}$ transcription, and the other through direct *Rb* transcription. These observations are consistent with the emerging idea that p53 regulates transcription of different genes, depending on the type of stimuli that provoked its activation (Zhao et al., 2000). Interestingly, the existence of a positively regulated p53-binding site on the *Rb* promoter has been known for several years (Osifchin et al., 1994), but no transcriptional induction of the *Rb* gene was found in apoptotic or growth-arresting situations, so far. Our results reveal the existence of a physiological condition in which p53 directly transactivates the *Rb* gene.

Recently, a family of p53-related proteins has been discovered. This family includes, besides p53, p73 and p63 proteins that share homology with the activation, the DNA-binding, and the oligomerization domains of p53. These proteins, when overexpressed in mammalian cells, can transactivate some of the p53 target genes and appear to be involved in differentiation and development pathways (Oren, 1997; Kaelin, 1999; Mills et al., 1999; Yang et al., 1999, 2000). The genetic analysis we performed by comparing the differentiation abilities of primary myoblasts explanted from $p53^{+/+}$ and $p53^{-/-}$ mice allowed us to distinguish the function of p53 from those of its family members, demonstrating that the role played by p53 in muscle differentiation is specific of this protein. This result suggests that p53 and its family members have no overlapping activities on differentiation and thus might not be functionally redundant during ontogenesis. These considerations leave open the discrepancy between impaired differentiation of p53 null muscle cells in vitro and the apparently normal muscle development in p53 null mice. Temporary, slight defects in muscle development cannot be ruled out since, to our knowledge, detailed developmental analyses of skeletal muscles have not yet been performed in $p53^{-/-}$ mice. On the other hand, an explanation for this discrepancy might be suggested by the observation that, in dnp53-expressing C2C12 cells, the inhibition of differentiation is present only when these cells are stimulated to differentiate by growth factor withdrawal (Soddu et al., 1996) (Fig. 7). It is known that skeletal muscle differentiation in vitro can be obtained by two different stimuli, growth factor deprivation and cell to cell contact (Martelli et al., 1994). We have found that cell confluence, even in the presence of serum, is able to increase pRb expression in a p53-independent manner (Coen, S., and S. Soddu, unpublished results). Differentiation-promoting stimuli due to cell to cell contact can be avoided in vitro, but not during in vivo development, suggesting that they can provide a functional replacement for p53. We do not know the molecular mediators of this effect nor whether it is relevant for development; nevertheless, the strong and frequent association between p53 alterations and the undifferentiated phenotypes described in many human cancers (Feinstein et al., 1991; Fagin, 1995) is in agreement with this hypothesis. Indeed, one peculiar characteristic of tumor transformation is the loss of contact inhibition. This would make tumor cells more sensitive than their normal counterparts to the absence of p53 in terms of differentiation capacity. If proved, this hypothesis might solve an apparent incongruity that has appeared between clinical and basic biological researches in the p53 field. While basic research has mostly studied the control of cell-cycle checkpoints and the apoptotic function of p53, a large number of clinical and epidemiological works have associated*TP53* gene alterations to loss of tumor differentiation (anaplasia). A commonly accepted model has it that genomic instability is the cause of anaplasia, through direct alterations of regulatory and structural differentiation genes. However, this hypothesis is in contrast with the differentiation-promoting effects of different compounds or of the exogenous expression of the wild-type p53 protein per se. Indeed, both approaches induce differentiation in tumor cells without correcting the genomic instability, suggesting a dual role for p53 in tumor development as gatekeeper (Levine, 1997) and differentiation controller. The finding that p53 regulates not only the cell-cycle progression function of pRb, but also its differentiation-promoting function, matches the *TP53* gene to the prototypic oncosuppressors, which are essential for embryonic development and play important roles in regulating cell cycle and differentiation.

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