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Letter to the Editor

p53 is involved in the differentiation but not in the differentiation-associated apoptosis of myoblasts

Dear Editor,

The p53 tumor suppressor and its recently discovered family members are involved in a variety of biological functions, including regulation of cell cycle control, apoptosis, and differentiation.[1–4] These proteins can have overlapping functions during these processes or they can work at the crossroad linking a network of signals that regulate the balance among these processes. To discriminate between these possibilities, we took advantage of the differentiation process of C2C12 myoblasts, which requires cell cycle withdrawal and occurs along with a certain degree of apoptosis. C2C12 myoblasts express wild-type p53 (wtp53) protein, which is activated during differentiation.[5,6] The interference with the endogenous wt-p53, by the expression of a dominant-negative p53 (dn-p53) protein, strongly inhibited terminal differentiation.[5] First, we investigated whether differentiation-associated apoptosis (DAA) and differentiation are mutually exclusive pathways, and therefore if there is the absence of simultaneous expression, in the same cell, of apoptosis and differentiation markers. To this aim, we analyzed, at single cell level, by double fluorescence staining, a series of differentiation-related proteins and the apoptosis-induced cleavage of genomic DNA by TUNEL assay. Upon differentiation induction by serum withdrawal, more than 50% of the TUNEL-positive C2C12 cells expressed myogenin, and about 40% expressed p21 and/or pRB (data not shown), indicating that DAA and early steps of C2C12 differentiation are not mutually exclusive.

It has been found that during C2C12 differentiation p53 shows a transient increment of stability, measurable by pulse-chase experiments,[6] and a transient surge of transcriptional activity.[5] However, we could never couple this p53 transient stability and activation with a p53-positive immunofluorescence staining (S Soddu, unpublished results). Thus, to evaluate p53 activity at single cell level in terminally differentiating and DAA C2C12 cells, we exploited the properties of GFP-based vectors to study the p53-specific transcriptional activity.[7] We cloned the 13 copies of the p53 consensus binding-sequence of the PG_{13}CAT reporter vector[8] upstream of the GFP gene (PG_{13}GFP). PG_{13}GFP-transduced C2C12 cells (C2-PG_{13}GFP) were selected and checked for response to p53 activation by Adriamycin (ADR)-induced DNA damage. In the presence of differentiation promoting medium, the appearance of green fluorescence in C2-PG_{13}GFP cells began after 24 h and reached its maximum after 48–72 h (Figure 1A). Morphological analysis of C2-PG_{13}GFP cells showed that GFP was present in all the differentiated C2C12 cells. Despite the long half-life of the GFP protein (more than 3 days, data not shown), no fluorescence was ever detectable in the DAA cells, at any time point, in both cell clones. This result indicates that p53 transcriptional activity belongs to the differentiating cells, while DAA seems to be independent of p53 activation.

To confirm the p53 transcriptional activity data obtained at single cell level, we separated apoptosing from differentiating cells by shake off and analyzed the two populations independently. C2-PG_{13}CAT cells and C2-MG_{15}CAT controls were cultured in a differentiation-promoting medium for 48 h, separated, incubated with PI, and quantified by cytofluorimetric analysis. Unstimulated, proliferating cells were chosen as a reference point in these experiments. As expected, proliferating C2-PG_{13}CAT cells had a basal chloramphenicol acetyltransferase (CAT) activity comparable to the C2-MG_{15}CAT control cells (data not shown). Absence of CAT activity was also found in DAA cells, while a fourfold induction of activity was consistently found in the differentiating population (Figure 1B). As positive control, DNA damage and apoptosis were induced by ADR treatment on C2-PG_{13}CAT cells (Figure 1B). Consistently, cytofluorimetric analysis of C2-PG_{13}GFP cells, obtained by ADR treatment or DAA purification, showed that only the ADR-treated cells were fluorescent (Figure 1C). Thus, the absence of p53-dependent transcriptional activity, in C2C12 cells undergoing DAA, is not due to a generic silencing of the reporter vectors, but reflects the absence of p53 involvement in this biological event.

The observation that DAA C2C12 cells can still be positive for $p21^{WAF1}$ and pRB, though they are negative for p53 activity, might depend on the fact that p53 activation is a critical step required for complete protection from DAA. Alternatively, p53 activation is downstream of the crossroad between differentiation and DAA. C2C12 cells expressing high levels of the temperature-sensitive p53Val135 mutant protein (tsp53)[9] were used to discriminate between these possibilities. No difference in the DAA rate was found between tsp53-expressing and the control cells at restrictive (dominant-negative activity) and permissive (wild-type activity) temperatures. This result demonstrates that p53 is not involved in the protection from DAA, but it is rather activated after the cell decision between differentiation and DAA.

The dn-p53 proteins, such as the p53Val135 mutant we used to interfere with the endogenous wt-p53 of C2C12 cells, are believed to work by forming mixed, transcriptional inactive oligomers between wild-type and mutant p53 proteins.[9] Recently, weak, but transcriptionally relevant heterotypic interactions have been described

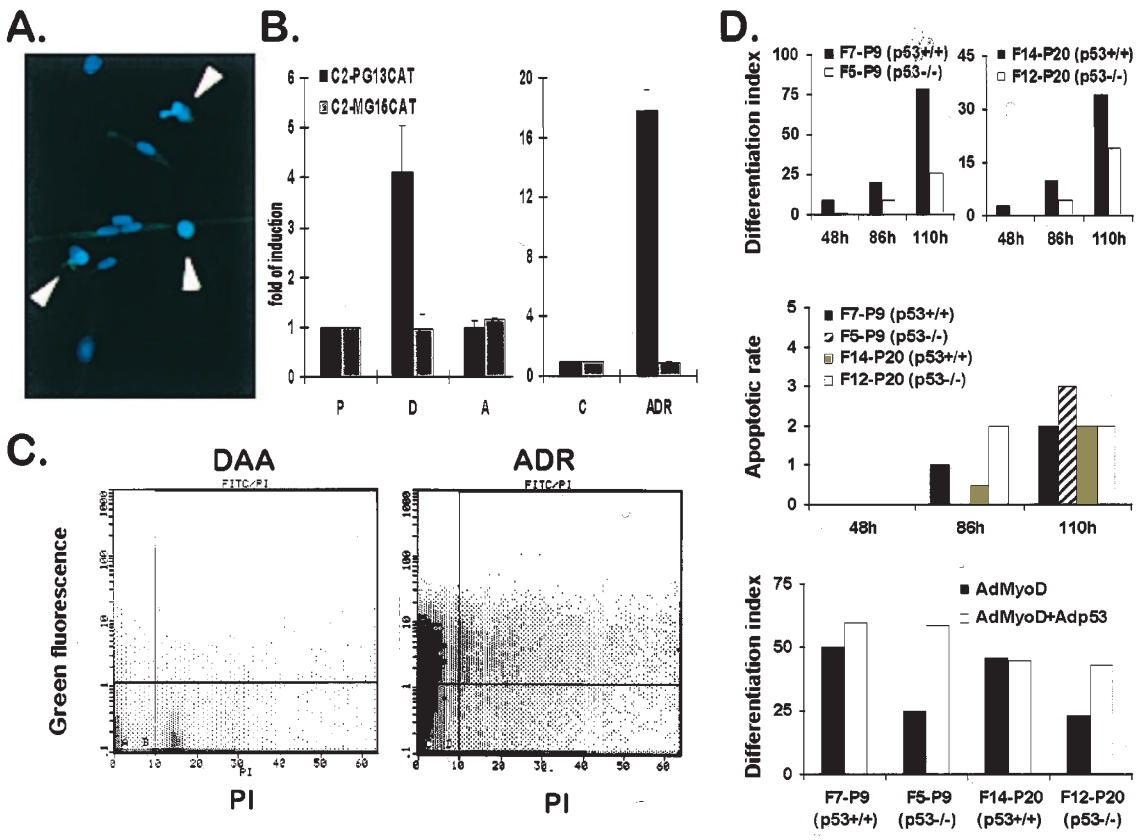

Figure 1 Analyses of p53 role in differentiation and DAA. (**A**) Detection of green fluorescence in C2-PG_{13}GFP cells 48 h upon induction of differentiation by serum withdrawal. Blue fluorescence depends on nuclei stained by Hoechst while green fluorescence depends on GFP expression. The white arrows indicate apoptotic cells. (**B**) CAT activity. C2-PG_{13}CAT and C2-MG_{15}CAT cells were maintained in growing medium, incubated in differentiation promoting medium for 48 h, or treated for 20 h with 1 μg/ml ADR before separation and analysis for CAT activity. The percentages of dead and live cells after shake off separation were evaluated by cytofluorimetric analysis, upon incubation of fresh cells with PI, which does not stain live cells in this condition. The analysis showed that PI-positive cells were 60–80% of the apoptotic population and 5–25% of the adherent one. P, indicates proliferating cells; D, differentiating cells; A, DAA cells; C, untreated cells; ADR, ADR-treated cells. Folds of induction are relative to the basal value of proliferating cells. (**C**) C2-PG_{13}GFP cells were prepared as described in (**B**). Cytofluorimetric analyses of DAA and ADR treated cells are reported. (**D**) Differentiation indices and apoptotic rates in MyoD-converted primary fibroblasts. $p53^{-/-}$ and $p53^{+/+}$ primary fibroblasts were obtained from two littermates at day 9 (P9) and 20 (P20) post-birth. These cells were infected with a recombinant adenovirus carrying the muscle regulatory gene *MyoD* and incubated in differentiation-promoting medium. Upper panel: differentiation indices were calculated with MyHC and Hoechst staining. Middle panel: apoptotic rates were measured by TUNEL assay. Lower panel: differentiation indices were measured as in the upper panel after co-infection of the fibroblasts with two recombinant adenoviruses carrying *MyoD* and *TP53* genes, respectively

between p73 and p63,[10] and between some mutant p53– such as p53His175, but not p53His273– and wild-type p73.[10,11] These findings raise the question whether the dn-p53Val135 mutant, that has been largely used to study the biological function of p53 protein, can interfere not only with the wt-p53 protein, but also with p53-family members. To answer this question, $p53^{-/-}$ and $p53^{+/+}$ primary fibroblasts were explanted from newborn mice. These cells were maintained in culture simultaneously and used only within the third passage in culture to avoid interference by senescence of $p53^{+/+}$ fibroblasts and immortalization of $p53^{-/-}$ fibroblasts. Myoblast conversion was obtained by infection with a recombinant adenovirus carrying the myogenic-determining factor MyoD.[12] As shown in Figure 1D, no significant difference between $p53^{-/-}$ and $p53^{+/+}$ cells was observed for DAA, while a strong reduction in differentiation capacity was found in the two $p53^{-/-}$ cell lines, compared to their relative $p53^{+/+}$ cell lines. This reduced differentiation ability was completely recovered by cell infection with a recombinant adenovirus carrying the *TP53* gene[5] (Figure 1D). These data confirm the previously indicated role for p53 in muscle differentiation obtained by employing dn-p53 proteins,[5,13] and demonstrate that this p53 role is not shared by other members of its family.

Altogether, these data show that p53 is not a regulator at the crossroad of signals balancing apoptosis and differentiation, at least in myoblasts, but it is activated downstream of myogenin, $p21^{WAF1}$ and pRB up-regulation, only in the differentiating cells. Furthermore, p53 differentiation activity is specific to this protein and does not overlap with that of other p53 family members.

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