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Human fibroblasts require the Rb family of tumor suppressors, but not p53, for PML-induced senescence

Frédérick A Mallette[1,2], Stéphane Goumard[1,2], Marie-France Gaumont-Leclerc[1], Olga Moiseeva[1] and Gerardo Ferbeyre*[,1,3]

[1]*Département de biochimie, Université de Montréal, Montréal, Québec, Canada H3C 3J7*

Cellular senescence is a permanent cell cycle arrest that can be triggered by a variety of stresses including short telomeres and activated oncogenes. Promyelocytic leukemia protein (PML) is a central component of the senescence response, and is able to trigger the process when overexpressed in human diploid fibroblasts (HDFs). Senescence induced by PML in HDFs is characterized by a modest increase in p53 levels and activity, the accumulation of hypophosphorylated Rb and a reduced expression of E2F-dependent genes. To dissect the p53 and Rb family requirements for PML-induced senescence, we used the oncoproteins E6 and E7 from human papillomavirus type 16. We found that the coexpression of E6 and E7 inhibited the growth arrest and senescence induced by PML. In addition, these viral oncoproteins blocked the formation of PML bodies and excluded both p53 and Rb from PML bodies. Expression of dominant-negative p53 alone failed to block PML-induced senescence and expression of E6 only delayed the process. On the other hand, expression of E7 was sufficient to block PML-induced senescence, while an E7 mutant unable to bind Rb did not. Together, these data indicate that PML-induced senescence engages the Rb tumor-suppressor pathway predominantly.
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Introduction

Human diploid fibroblasts (HDFs) respond to a variety of stresses by entering into a metabolically active cell cycle arrest characterized by flat morphology and specific changes of gene expression (Campisi, 2001; Serrano and Blasco, 2001). This phenotype, known as cellular senescence, was first observed after serial passage of HDFs in culture (Hayflick and Moorhead, 1961). Senescence can also be induced by oncogenes and DNA-damaging agents, suggesting that it plays a role as a tumor-suppressor mechanism, blocking the expansion of cells with the potential of becoming malignantly transformed (Campisi, 2001; Serrano and Blasco, 2001). Consistent with this idea, establishment of the senescent arrest in response to a variety of stressors depends on the p53 and the Rb tumor-suppressor pathways (Campisi, 2001; Serrano and Blasco, 2001). Accordingly, most genes found to regulate senescence (i.e. p21, ING1, $p16^{INK4a}$) can be placed either upstream or downstream of p53 or the Rb family of tumor suppressors (Hara *et al*., 1996; Garkavtsev and Riabowol, 1997; Palmero *et al*., 1997; Chang *et al*., 2000; Young and Smith, 2001; Macip *et al*., 2002).

Definitive proof for the involvement of the Rb and the p53 tumor-suppressor pathways in senescence has been obtained in cells from different knockout mice (Serrano *et al*., 1997; Dannenberg *et al*., 2000; Sage *et al*., 2000; Lin and Lowe, 2001; Peeper *et al*., 2001). However, for technical reasons, similar experiments have not been published so far in human cells. Hence, the role of the p53 and Rb tumor-suppressor pathways in human cells has been inferred from correlative evidence or the use of several viral oncoproteins that inactivate these tumor-suppressor pathways (Campisi, 2001). For example, simultaneous inhibition of both p53 and Rb by the viral oncoproteins, large T from SV40, E1A from adenovirus or E6/E7 from papillomavirus can inhibit the senescent arrest triggered by oncogenes and delay the arrest activated by short telomeres in human fibroblasts and keratinocytes (Serrano *et al*., 1997; Kiyono *et al*., 1998; Hahn *et al*., 2002; Rheinwald *et al*., 2002).

Recent results indicate that the control of cellular senescence is different between human and mouse cells, suggesting that the results obtained in a mouse model should not be extrapolated to human cells. For example, in HDFs, serial passage induces senescence through telomere shortening, a process that provides a signal mimicking broken DNA ends (Harley *et al*., 1990; Greider, 1998; Sedivy, 1998). In mouse fibroblasts, serial passage triggers senescence not due to telomere attrition but due to the stress of the culture conditions (culture shock) (Sherr and DePinho, 2000). Moreover, induction of senescence by oncogenic *ras* or telomere deprotection requires p53 in mouse cells but not in HDFs (Serrano *et al*., 1997; Serrano and Blasco, 2001; Smogorzewska and de Lange, 2002).

*Correspondence: G Ferbeyre, Département de biochimie, Université de Montréal, E-515, C.P. 6128, Succ. Centre-Ville, Montréal, Québec, Canada H3C 3J7; E-mail: g.ferbeyre@umontreal.ca
[2]These two authors contributed equally to this work
[3]Delivery address: Département de Biochimie, Université de Montréal, Pavillon Principal, A-533, 2900 Édouard Montpetit, Montréal, Québec, Canada H3T 1J4
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Senescence induced by short telomeres or oncogenic *ras,* both in mouse and human cells, activates another candidate tumor suppressor known as promyelocytic leukemia protein or PML (Ferbeyre *et al*., 2000; Pearson *et al*., 2000). PML was originally discovered as a translocation partner of the RARα gene in patients with acute promyelocytic leukemia (reviewed in Melnick and Licht, 1999; Salomoni and Pandolfi, 2002). Enforced expression of PML is sufficient to trigger senescence both in human and mouse fibroblasts, suggesting that it is an important component of the senescence pathway. In mouse fibroblasts, p53 is essential for PML-induced senescence and colocalizes with PML and CBP into PML bodies (Pearson *et al*., 2000), also known as PODs for PML oncogenic domains. PML-induced senescence in HDFs was accompanied by a modest increase in p53 levels, activity and several post-translational modifications of p53 (Ferbeyre *et al*., 2000; Pearson *et al*., 2000; Bischof *et al*., 2002). In addition, PML-induced senescence was blocked by enforced expression of the p53 deacetylase SIRT1 (Langley *et al*., 2002). Together, these reports suggest that in human cells p53 functions are also important for PML-induced senescence, but they do not attest whether p53 is actually required for the process. PML-induced senescence in HDFs is also characterized by the accumulation of hypophosphorylated Rb and the colocalization of a fraction of the Rb protein into PML bodies (Ferbeyre *et al*., 2000). Again, it is not known whether the Rb pathway is essential for the establishment of PML-induced senescence.

Here we study the p53 and the Rb family requirements for PML-induced senescence in human cells by using the human papillomavirus type 16 (HPV16) proteins E6 and E7. E6 promotes p53 degradation through the proteasome (reviewed by Mantovani and Banks, 2001), while E7 binds to pocket proteins (Rb, p107, p130) and interferes with their ability to block E2F-dependent gene expression (reviewed by Munger *et al*., 2001). We show that coexpression of E6 and E7 efficiently blocked PML-induced senescence. Surprisingly, expression of E7 alone was also sufficient to bypass PML-induced senescence, while expression of E6 or dominant-negative p53 did not. The ability of E7 to bypass PML-induced senescence required binding and inactivation of pocket proteins because a well-known mutant of E7 unable to bind these proteins was also inactive for blocking PML-induced senescence. Together, these results contrast those published using mouse fibroblasts and suggest that in HDFs, PML-induced senescence predominantly engages the Rb tumor-suppressor pathway.

Results

Expression of E6 and E7 blocks PML-induced senescence in human diploid fibroblasts

To investigate whether PML-induced senescence requires p53 and Rb family functions, we introduced the viral oncoproteins E6 and E7 into the human diploid fibroblasts IMR90. The populations of cells expressing E6/E7 or the empty vector LXSN were then infected with retroviruses that express PML, oncogenic Ras or a control vector. As previously reported (Ferbeyre *et al*., 2000; Pearson *et al*., 2000), PML-expressing cells arrested proliferation shortly after selection (Figure 1a, b) and remained arrested with features of cellular senescence, that is, a flat cell morphology and positive staining for the senescence-associated β-galactosidase (SA-β-gal) (Figure 1c, d). However, expression of PML in HDFs expressing E6/E7 did not lead to cell cycle arrest or senescent features (Figure 1). In contrast to PML, Ras-V12 induced a permanent growth arrest in IMR90 cells expressing either the control vector LXSN or E6/E7 (Figure 1a, c, d). On the other hand, the percentage of SA-β-gal-positive cells induced by Ras-V12 was significantly lower in cells expressing E6/E7, suggesting that these viral oncoproteins partially inhibited the senescent program induced by oncogenic *ras* without affecting the cell cycle arrest (Figure 1c, d). Accordingly, SV40 large T, which also inactivates p53 and Rb, requires perturbation of protein phosphatase 2A by SV40 small t to completely rescue cells from Ras-induced senescence (Hahn *et al*., 2002).

As reported previously, we found that expression of Ras-V12 or PML in HDFs increased the number and size of PODs (Figure 1e, Table 1). In cells expressing E6/E7, the number of PODs was reduced from around 26 in cells expressing Ras-V12 or PML to 13–14 PODs per cell, which is the number typically found in growing fibroblasts (Figure 1e, Table 1). Enforced expression of PML or Ras-V12 increased the size of PODs, and this increase was also inhibited in cells expressing E6/E7 (Table 1). Hence, E6 and E7 were sufficient to fully bypass the senescent cell cycle arrest induced by enforced expression of the PML protein.

The colocalization of p53 and Rb with PML is impaired in cells expressing E6 and E7

During senescence induced by short telomeres, Ras-V12 or PML, a fraction of the tumor-suppressor proteins p53 and Rb localize to PODs (Ferbeyre *et al*., 2000; Pearson *et al*., 2000). To investigate whether the viral oncoproteins E6 and E7 alter the functions of PODs during senescence, we studied the levels and localization of PML, Rb and p53 in HDFs expressing these oncoproteins. In cells expressing PML, we observed that staining for endogenous p53 also stained the PML bodies. Hence, most visualized PML bodies contained p53 immunoreactive material (Figure 2a).

Similarly, we found that a significant fraction of endogenous Rb localized with PML into PODs (Figure 2b). Moreover, we noticed that approximately 16% of the cells displayed very large PML bodies (mean size of 150 pixels), doubling the size of the PODs seen in the rest of the cells, which have a size of approximately 75 pixels. In cells with large PODs, the anti-Rb antibody was able to stain the PODs like the anti-PML antibody (Figure 2b third lane). The association of p53 and Rb

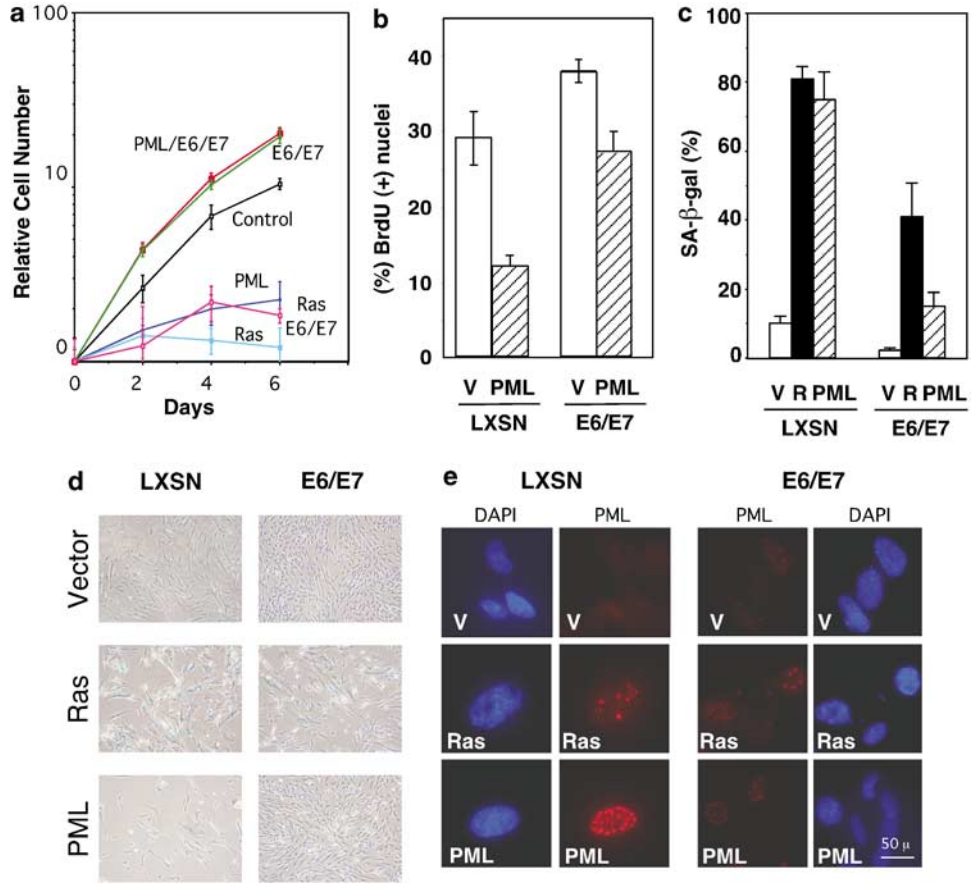

Figure 1 Inhibition of PML-induced senescence by retroviral-mediated expression of HPV E6/E7. (**a**) Growth curves: IMR90 cells expressing PML, PML plus E6/E7, Ras-V12, Ras-V12 plus E6/E7, E6/E7 and only control vectors (Control) were plated as described in Materials and methods. Cell numbers were scored immediately after attachment or after 2, 4 and 6 days in culture. (**b**) BrdU incorporation assay: the same cell populations were plated at day 6 of the growth curve and grown in the presence of BrdU for 3 h. The graphic shows the average of nuclei positive for BrdU incorporation after counting 200 cells in three separate experiments. (**c**, **d**) SA-β-gal staining: cells were fixed and stained at a day corresponding to day 6 of the growth curve. The graph in (**c**) shows the average of cells that stained positive for SA-β-gal after counting 200 cells in three separated experiments. (**e**) Immunodetection of PML bodies: cells infected with the control vector LXSN or its derivative containing E6/E7 were infected with either another control vector or its derivatives expressing Ras-V12 or PML. Cells containing these retroviruses were fixed at a day corresponding to day 6 of the growth curves. PML bodies were visualized by indirect immunofluorescence using the anti-PML antibody PG-M3. Nuclei were counterstained with DAPI

Table 1 E6/E7 reduce the number of PML bodies in cells expressing oncogenic *ras* or PML-IV

	PML bodies: number[a]	*PML bodies: area*[b]
LXSN/vector	9±4	35±18
LXSN/Ras-V12	26±10	48±20
LXSN/PML-IV	26±11	117±36
E6/E7/vector	8±4	20±10
E6/E7/Ras-V12	14±10	34±24
E6/E7/PML-IV	13±8	36±28

[a]The number of PML bodies is the average of counting 100 cells for each cell population. [b]The area of the PML bodies was estimated using the morphometric analysis feature of the MetaMorph software. We obtained the total quantity of pixels per nucleus, using an intensity threshold that lighted all PML bodies in a nucleus. This number was then divided by the amount of PML bodies present in the cell. Thus, the relative size of the PML bodies can be estimated, expressed in pixels

with PML bodies was not observed in cells expressing E6 and E7 (Figure 2a, b). Few PML bodies were observed in these cells and none of them contained p53 or Rb immunoreactive material, even in pictures taken with longer expositions. To better show the loss of the colocalization of p53 and Rb with PML in cells expressing E6/E7 and PML, we enlarged and deconvoluted the frames corresponding to p53 and Rb staining in these cells and the corresponding controls (Figure 2c). Both p53 and Rb show a granular pattern of staining in the control cells that corresponds in part with the PML staining seen in Figures 2a, b. In cells expressing E6 and E7, the p53 and Rb staining is largely homogenous. Therefore, in cells expressing E6 and E7, PML does not form nuclear bodies as efficiently as in normal cells and p53 and Rb are evenly localized in the nucleus.

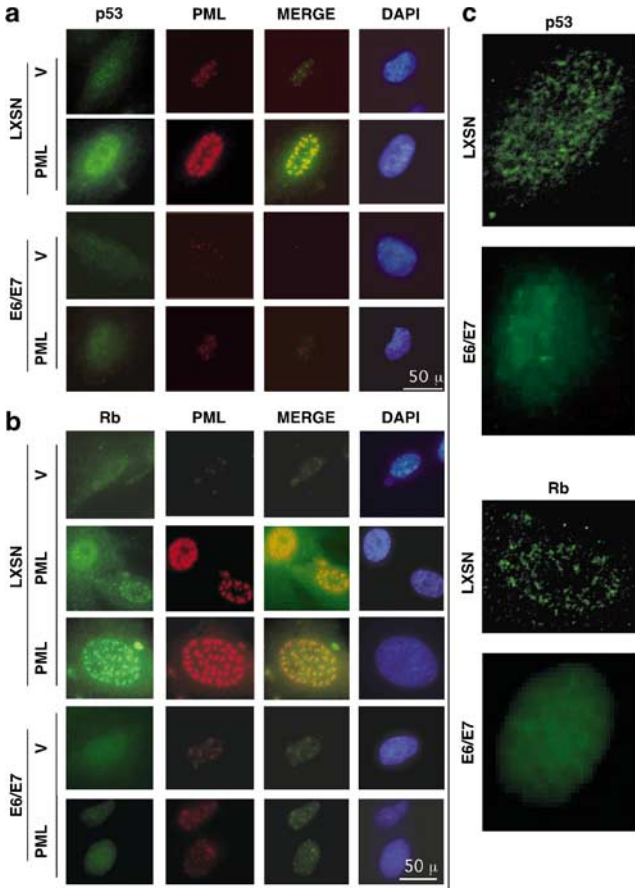

Figure 2 Effects of E6/E7 on p53 and Rb localization during PML-induced senescence. Cells expressing the control vector LXSN or its derivative encoding for E6/E7 were infected with either another control vector or its derivative expressing PML. (**a**) p53 protein and PML bodies were visualized by indirect immunofluorescence using the anti-PML antibody (PG-M3) and the anti-p53 antibodies (1801 and 421). (**b**) Rb protein was visualized by indirect immunofluorescence using the anti-Rb antibody Rb1C1 and PML bodies were visualized as in (**a**). Two kinds of cells are shown to represent the population of cells expressing PML and the control vector LXSN. The middle panel represents the majority of the cells, while the bottom panel is characteristic of around 16% of the cells (see text for comments). (**c**) The frames corresponding to p53 staining in PML-expressing cells without (second from the top in panel a) and with E6/E7 (fourth from the top in panel a) and corresponding to Rb staining in PML-expressing cells without (cell to the right in second frame from the top in panel b) and with E6/E7 (fifth from the top in panel b) were enlarged and deconvoluted (Metamorph) to show the details of p53 and Rb localization

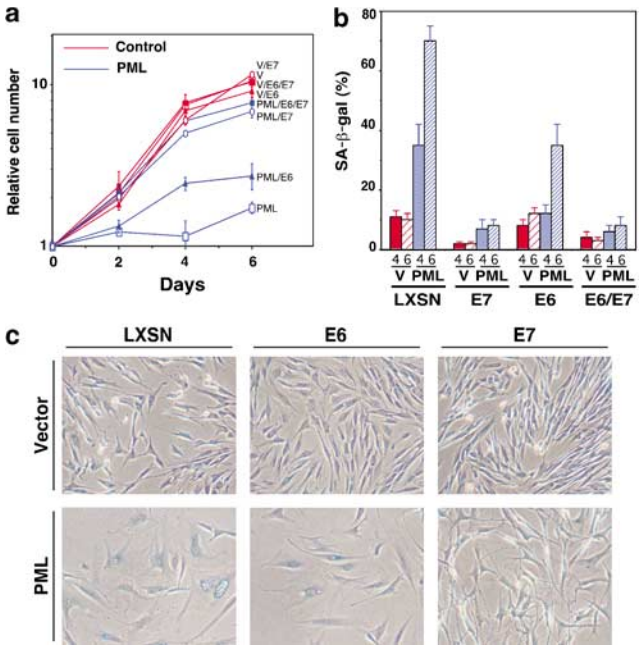

Figure 3 Individual expression of E7 is sufficient to inhibit PML-induced senescence. (**a**) Growth curves: IMR90 cells expressing the control vector LXSN or its derivative expressing E6, E7 or E6/E7 were infected with a control vector pLPC (red) or its derivative expressing PML (blue). Infected populations were plated for growth curves 2 days after selection. Cell numbers were scored immediately after attachment or after 2, 4 and 6 days in culture. (**b**, **c**) SA-β-gal staining: The graph in (**b**) shows the average of cells that stained positive for SA-β-gal at days 4 and 6, scored by counting 200 cells in three separated experiments

E7 rescues HDFs from PML-induced senescence

To investigate the relative contribution of the p53 and the Rb pathways to PML-induced senescence, we coexpressed PML with either E6 or E7 in HDFs. First, we infected HDFs with the LXSN (control vector) or its derivatives LXSNE6 or LXSNE7. Populations infected with these viruses were then infected with pLPC retrovirus or its derivative expressing PML. In cells with the empty vector, PML induced a growth arrest (Figure 3a) with the characteristics of cellular senescence (Figure 3b, c). In cells expressing E6, PML-induced senescence was delayed (Figure 3b) and the number of cells expressing SA-β-gal was reduced. Hence, E6 partially suppressed the senescence phenotype induced by PML. In cell populations coexpressing E7 and PML, growth arrest did not occur (Figure 3a), but the growth rate was slightly slower than in cells expressing E7 alone or cells expressing PML with both E6 and E7. This reduction in growth correlated with the ability of E7 to induce p53 and cell death (see Figure 6 below and supplementary Figure S1). Thus, E7 but not E6 rescues HDFs from PML-induced senescence. We also studied the individual effect of E6 and E7 on PML bodies. Neither E6 nor E7 was able to reduce the ability of PML to form PODs (supplementary Figure S2 and Table SI). Therefore, E7 rescues PML-induced senescence by acting downstream or at the level of PML bodies.

E7 is mainly known for its ability to inhibit the functions of the Rb family of tumor suppressors. However, E7 binds other targets (Munger *et al*., 2001) and, in principle, its ability to inhibit PML-induced senescence might be Rb-independent. To evaluate whether Rb binding by E7 is essential to inhibit PML-induced senescence, we used a well-known E7 mutant (E7Δ21–24), unable to bind the Rb family of pocket proteins but conserving intact other functions of E7 (Helt and Galloway, 2001). Cells expressing this mutant entered senescence in response to PML with higher efficiency than wild-type HDFs, according to a

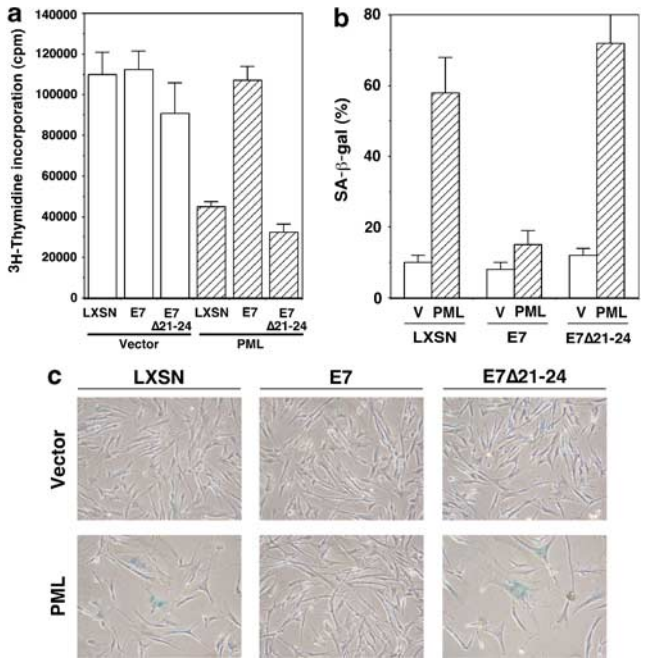

Figure 4 The ability of E7 to bind Rb is required for the inhibition of PML-induced senescence. (**a**) ^{3}H-thymidine incorporation assay: IMR90 cells expressing the control vector LXSN or its derivative expressing E7 or E7Δ21-24 were infected with a control vector pLPC (open bars) or its derivative expressing PML (ruled bars). Cells (2×10^4) were plated in triplicate 6 days after selection; 24 h later, a pulse of 5 μCi of ^{3}H-thymidine was added and the incorporation was scored after overnight incubation (**b**). (**c**) SA-β-gal staining as in Figure 3

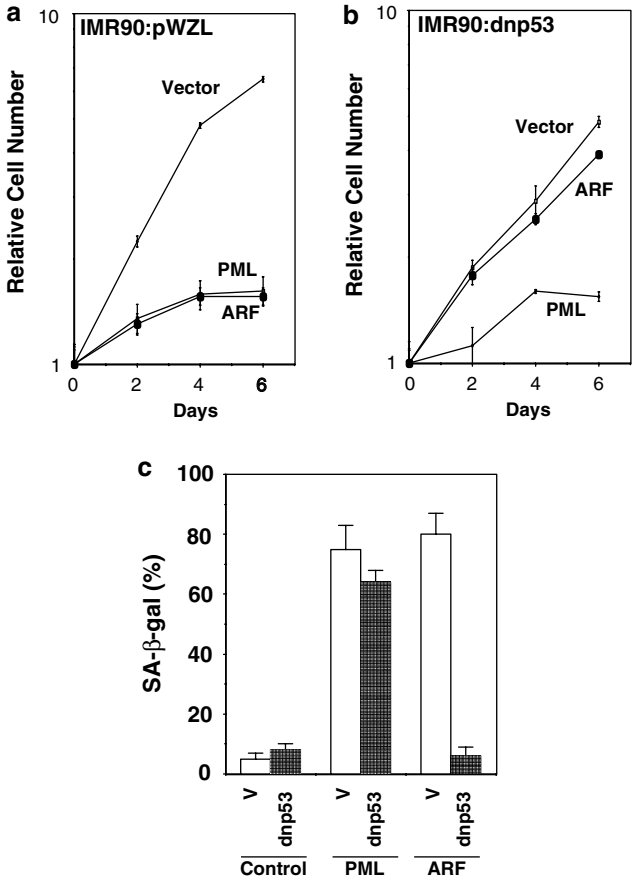

Figure 5 Dominant-negative p53 is not sufficient to inhibit PML-induced senescence. (**a**, **b**) Growth curves: IMR90 cells expressing pWZL vector (**a**) or its derivative coding for dominant-negative p53 (dnp53) were infected with pLPC (Vector) pLPCPML (PML) or pLPCp14^{ARF} (ARF), and plated as described in Materials and methods. Cell numbers were scored immediately after attachment (day 0) or after 2, 4 and 6 days in culture. The plot shows the cell number relative to the initial value for every cell population. (**c**) SA-β-gal: cells at a day corresponding to day 6 of the growth curve were fixed and stained as described in Materials and methods. The graph shows the average of cells that stained positive for SA-β-gal after counting 200 cells in three separated experiments

^{3}H-thymidine incorporation assay (Figure 4a) that measures cell cycle arrest, and according to the SA-β-gal assay that measures senescence (Figure 4b, c). Therefore, the ability of E7 to bypass PML-induced senescence requires inactivation of the Rb tumor-suppressor pathway.

A dominant-negative p53 does not block PML-induced senescence

Although E6 was unable to rescue PML-induced senescence, it was able to delay the cell cycle arrest and to reduce the number of cells stained for SA-β-gal. This could be secondary to inactivation of p53 or another target of E6 (Mantovani and Banks, 2001). To clear this point, we further examined the role of the p53 pathway in PML-induced senescence, by using a dominant-negative p53 allele *p53175H* (dnp53). Expression of PML in cells expressing dnp53 induced a cell cycle arrest with the characteristics of cellular senescence, which was indistinguishable from the senescence observed in control cells (Figure 5a, b). To verify that dnp53 was effectively blocking p53, we infected the cells expressing dnp53 with a p14^{ARF}-expressing retrovirus. In control IMR90 cells with an empty vector (pWZLHygro), p14^{ARF} induced a premature senescence accompanied with high p53 activity (Figure 5) (Stott *et al*., 1998). However, in cells expressing dnp53, p14^{ARF} failed to induce senescence (Figure 5). Together, these results suggest that p53 is not required for PML-induced senescence and that the effect of E6 in delaying the process is probably related to the inhibition of other targets by E6.

Cell cycle regulators in PML-induced senescence and in cells expressing HPV oncoproteins

Since E6 and E7 are known for their ability to disrupt the functions of p53 and the Rb family, respectively, we investigated the state of these tumor suppressors in cells expressing PML alone or in combination with E6, E7 or E6/E7 (Figure 6). Expression of PML in IMR90 cells bearing the control vector LXSN caused a modest but reproducible increase in p53 levels and its downstream target p21 (Figure 6, lanes 1 and 2). As expected, expression of E6/E7 triggered a dramatic reduction of

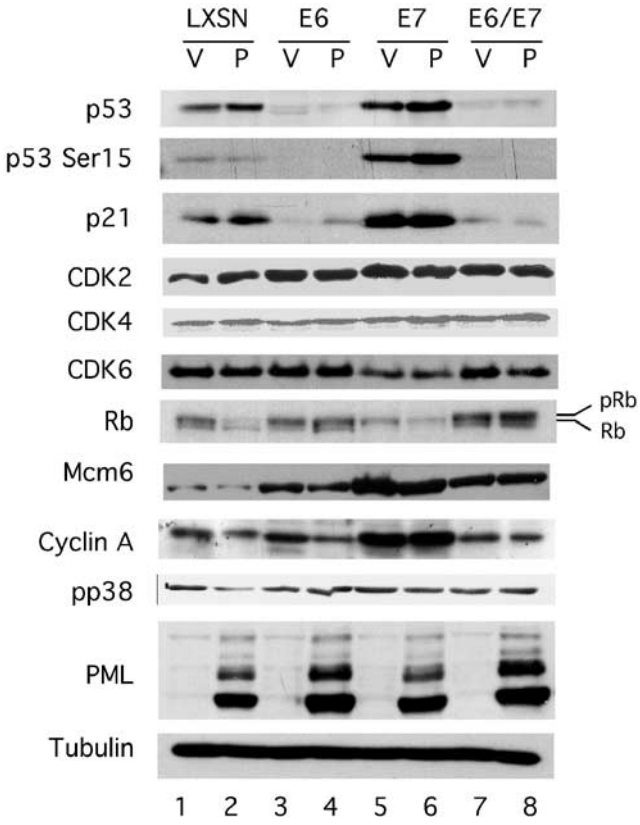

Figure 6 Effects of E6 and E7 on cell cycle regulators in HDFs expressing PML or a control vector. Cells expressing the control vector LXSN or its derivatives expressing E6, E7 or E6/E7 were infected with either another control vector or its derivative expressing PML. Cell extracts were prepared from cells collected at a day corresponding to day 6 of the growth curves. Total proteins (20 μg) were used for SDS–PAGE and individual proteins were visualized after immunoblotting using specific antibodies

p53 and p21 levels, both in control cells and in cells expressing PML (lanes 7 and 8). Expression of E6 alone was sufficient to reduce p53 levels and induction of p21 (lanes 3 and 4). In contrast, E7-expressing cells displayed high levels of p53 and p21 that were further increased in PML-expressing cells (lanes 5 and 6). The high p53 levels and activity in E7-expressing cells are consistent with the increased frequency of cell death observed in these cells (supplementary Figure S1). This was also observed in cells expressing E7Δ21–24 (data not shown). On the other hand, this p53 response in E7-expressing cells does not interfere with the ability of E7 to block PML-induced senescence. Expression of PML in IMR90 cells with the empty vector LXSN resulted in an important reduction of the hyperphosphorylated form of Rb (pRb) (lane 2) and an accumulation of the hypophosphorylated form (lanes 2 and 4). Proliferating cells predominantly displayed the hyperphosphorylated form of Rb (lanes 1, 3 and 5–8). This is not due to a reduction in the levels of any of the kinases known to phosphorylate Rb (CDKs 2, 4 and 6), since their levels were not affected by PML. The lower levels of Rb protein observed during senescence induced by PML (lane 2 *vs* 1) might reflect the fact that hypophosphorylated Rb is normally attached to an insoluble nuclear fraction and is thus, very difficult to solubilize. Low levels of Rb protein in total cell extracts were also reported during senescence induced by Ras, Raf or PML (Serrano *et al*., 1997; Ferbeyre *et al*., 2000). Hence, PML engages the Rb pathway by promoting the accumulation of the active hypophosphorylated form of Rb and expression of E6/E7 or E7 alone blocked the ability of PML to reduce Rb phosphorylation.

For additional characterization of the Rb pathway, we measured the levels of the E2F target genes Cyclin A and Mcm6. As expected, PML reduced the levels of these proteins in control normal fibroblasts (lanes 1 and 2). PML also reduced the levels of Cyclin A and Mcm6 in cells expressing E6 (lanes 3 and 4), but failed to do so in cells expressing E7 or E6/E7 (lanes 5 to 8). Note that these two proteins are highly upregulated in cells expressing E7. These results are consistent with the idea that these genes are repressed in an Rb family-dependent manner during PML-induced senescence. Importantly, since Mcm6 is an E2F-target gene expressed in G1, its levels reflects better the Rb-dependent repression in this stage of the cell cycle (Heidebrecht *et al*., 2001). Finally, PML-induced senescence is not accompanied by activation of the p38 MAP kinase (Figure 6), as observed during *ras*-induced senescence (Haq *et al*., 2002; Wang *et al*., 2002; Iwasa *et al*., 2003). This is in agreement with the idea of PML being donwnstream of both Ras and p38 MAP kinase in the senescence-signaling pathway activated by Ras. Therefore, both the genetic studies and the biochemical analysis indicate that PML-induced senescence in human cells predominantly engages the Rb tumor-suppressor pathway.

Discussion

Here we report that the oncoproteins E6 and E7 from HPV16 block the senescence program induced by PML. The effects of E6/E7 are thus reminiscent of those previously observed with the E1A oncoprotein from adenovirus, which also blocks senescence in response to oncogenic Ras or PML (Serrano *et al*., 1997; Ferbeyre *et al*., 2000). E1A shares with E7 the ability to bind and disrupt the functions of the Rb family proteins (pRb, p107 and p130) and cooperates with oncogenic *ras* to transform primary cells (Phelps *et al*., 1988). E1A shares with E6 an ability to inhibit p53, although through a distinct mechanism. E1A opposes p53 activity by binding to the p53 coactivator p300 (Lill *et al*., 1997), while E6 leads to p53 degradation (Mantovani and Banks, 2001). One advantage of the HPV oncoproteins over E1A to dissect the functions of tumor-suppressor pathways is that the relative contributions of the Rb and the p53 pathways can be easily studied by expressing E6 and E7 separately. Surprisingly, we found that expression of E7 alone, but not of E6 or of a dominant-negative p53, was sufficient to block PML-induced

senescence, indicating that in human fibroblasts, the p53 pathway is not essential for PML-induced senescence. Given the extensive literature implicating p53 in the senescent response of fibroblasts (Gollahon *et al*., 1998; Campisi, 2001), our results were surprising. However, p53 was reported to be nonessential for the senescent response of human fibroblasts to oncogenic *ras* (Serrano *et al*., 1997) or telomere deprotection (Smogorzewska and de Lange, 2002). Also, p53 null human cells from patients with Li–Fraumeni syndrome are not defective in replicative senescence (Medcalf *et al*., 1996). Together, these data support our conclusion that p53 is not required for the senescent cell cycle arrest of HDFs in response to enforced expression of PML.

The ability of E7 to bypass PML-induced senescence seems to be the result of blocking the Rb pathway. Correspondingly, an E7 mutant known for its defect in binding and blocking the Rb family of proteins was unable to inhibit PML-induced senescence. Paradoxically, in HDFs, we found that E7 induced p53 levels and activity and this was accompanied by increased cell death. However, despite this p53 response, cells expressing E7 did not arrest cell proliferation. This could be explained by the ability of E7 to promote an Akt-dependent phosphorylation of p21, preventing its nuclear localization and functions (Westbrook *et al*., 2002). Also, the Rb pathway seems to function downstream the p53 pathway in cell cycle regulation, as demonstrated in mouse cells with genetic ablation of all the three pocket proteins. These cells failed to enter senescence after serial passage or enforced expression of oncogenic *ras* despite the presence of activated p53 (Dannenberg *et al*., 2000; Sage *et al*., 2000; Peeper *et al*., 2001). Of interest, HDFs deficient of the Rb regulator $p16^{INK4a}$ are resistant to Ras-induced senescence (Brookes *et al*., 2002), and it would be relevant to study whether these cells can enter senescence in response to PML. Since PML is downstream of Ras in Ras-induced senescence, collectively, these data suggest a predominant role for the Rb pathway over the p53 pathway in PML-induced senescence in human cells. In agreement, reintroduction of Rb into Rb-deficient tumor cell lines induced a senescent cell cycle arrest in the absence of functional p53 (Xu *et al*., 1997).

In Figure 7, we propose a model that takes into account the results coming from studies on both mouse and human cell lines. In murine fibroblasts, the p53 and the Rb pathways are both essential for Ras- or PML-induced senescence, and as proposed before (Dannenberg *et al*., 2000; Sage *et al*., 2000; Peeper *et al*., 2001), p53 is upstream the Rb family of proteins. Hence, blocking either p53 or the Rb family of proteins bypasses senescence in mouse cells. In human cells, the contribution of the p53 pathway is not essential and both Ras (Serrano *et al*., 1997; Ferbeyre *et al*., 2000; Brookes *et al*., 2002) and PML can engage a senescent program that requires an intact Rb pathway. This model does not deny a role for p53 as a tumor suppressor against oncogenic stimuli or the ability of p53 itself to regulate senescence. In fact, the transformation of human primary cells by oncogenic *ras* clearly requires

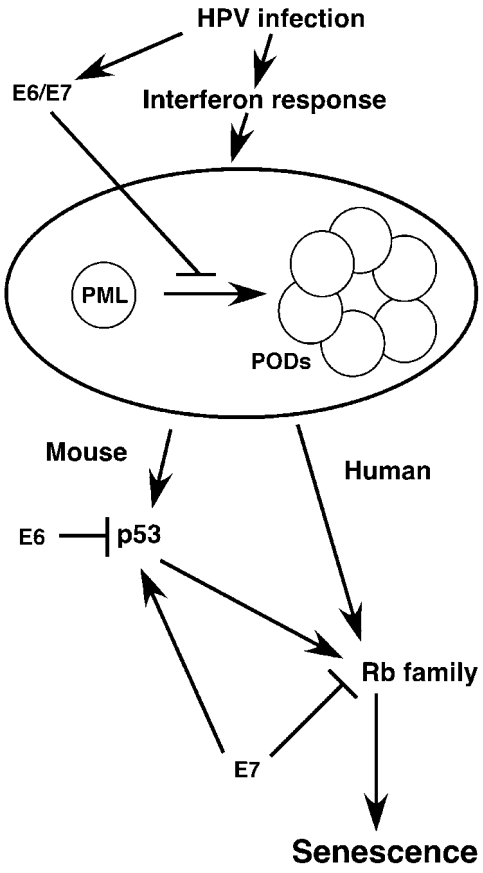

Figure 7 PML-induced senescence in mouse and human normal fibroblasts. In mouse cells PML induces senescence through p53, while the Rb family of proteins act downstream of p53, as suggested by Sage *et al.* (2000). In human fibroblasts, PML can directly engage the Rb pathway without a p53 requirement. HPV E7 blocks PML-induced senescence by blocking the Rb family of proteins, but also triggers a p53 response. E6 is then required to counteract E7-induced p53 activity. E6 and E7 also cooperate to inhibit the formation of PML bodies. E6 and E7 could overcome an interferon-PML senescent response aimed to restrain proliferation of cells infected by viruses

the inhibition of p53 functions (Hahn *et al*., 2002), and both Ras and PML induce p53 activity in these cells (Serrano *et al*., 1997; Ferbeyre *et al*., 2000; Pearson *et al*., 2000). Also, $p14^{ARF}$, E2F1 and β-catenin required an intact p53 pathway to induce senescence (Stott *et al*., 1998; Dimri *et al*., 2000; Damalas *et al*., 2001). However, the model does suggest that the senescent phenotype is a direct consequence of the action of the Rb family of tumor suppressors, perhaps due to their ability to repress multiple genes required for cell proliferation by promoting stable changes in their chromatin (Brehm *et al*., 1998; Brehm and Kouzarides, 1999; Nielsen *et al*., 2001).

It remains an open question as to how PML engages the Rb pathway to induce senescence. It has been reported that PML, like oncogenic Ras, can increase $p16^{INK4a}$ protein levels. However, high p16 levels in PML-expressing cells were only detected in cells already senescent (10 days after introduction of PML) (Ferbeyre

et al., 2000), but not at earlier time points (Pearson *et al*. (2000) and our data not shown). Therefore, $p16^{INK4a}$ might have a late role in PML-induced senescence, perhaps in maintaining the arrest. It has been also reported that PML blocks the transport of specific mRNAs from the nucleus to the cytoplasm. One of these PML-regulated transcripts is the one coding for cyclin D. As a consequence, cells with high levels of PML do not express cyclin D (Cohen *et al*., 2001). This mechanism deserves validation in conditions in which PML induces senescence. One additional explanation, supported by our Rb localization results is that the PML bodies provide a localized anti-CDK function that either protects Rb from phosphorylation or actively dephosphorylates Rb. Retroviral expression of CDK4 does not rescue PML-induced senescence (data not shown), and this would be consistent with either a defect in cyclin D mRNA export or the Rb localization model.

HPV is the single most important risk factor for developing cervical neoplasia (Franco *et al*., 2001). Our results suggest that a senescence response controlled by PML could be an important barrier to prevent the establishment of cervical carcinoma. Consistent with this idea, blocking the expression of E6 and E7 in cell lines derived from cervical carcinoma re-establishes the functions of p53 and Rb and induces senescence (Goodwin *et al*., 2000; Wells *et al*., 2000). Since PML is a critical regulator of senescence, studying the status of PML could be useful to predict the outcome of HPV infections of the cervix or the prognosis of premalignant cervical lesions.

Materials and methods

Cells and retroviruses

Normal HDF IMR90 cells (ATCC) were cultured in Dulbecco's modified Eagle medium (DMEM, GIBCO) supplemented with 10% fetal bovine serum (FBS, Hyclone, UT, USA) and 1% penicillin G/streptomycin sulfate (GIBCO). Retroviral-mediated gene transfer was performed as previously described (Ferbeyre *et al*., 2000). Infected populations were prepared as follows: IMR90 cells were first infected with retroviruses expressing E6/E7, E6, E7, E7Δ21–24 or an empty vector (LXSN), and incubated in geneticin (Gibco, 400 μg/ml) for 7 days to eliminate uninfected cells. Infected cell populations were then reinfected with retroviruses expressing oncogenic *ras* (H-Ras-V12) (Serrano *et al*., 1997), *PML-IV* (Ferbeyre *et al*., 2000) or an empty control vector (pBabe), and selected in puromycin 2.5 μg/ml for 3 days.

Cell proliferation and senescence determination

Various assays were used to determine cell proliferation. For growth curves, we collected infected cell populations 2 days after selection and seeded 2×10^4 cells in each well of a 12-well plate (Costar, Corning Inc., NY, USA). Cells were counted immediately after attachment (day 0) or on days 2, 4 and 6 after plating. Cell death was scored using the Trypan blue staining assay. BrdU and ^{3}H-thymidine incorporation assays were performed as described (Ferbeyre *et al*., 2000).

Senescence was scored by determining the percentage of population exhibiting a SA-β-gal activity, as described previously (Dimri *et al*., 1995).

Protein expression

To prepare total cell extracts, cells were collected by trypsinization, washed with PBS, lysed in 100 μl of SDS sample buffer (60 mM Tris-HCl pH 6.8, 10% glycerol, 2% SDS and 5% 2-mercaptoethanol) and boiled for 5 min. For immunoblotting, 20 μg of total cell protein were separated on SDS–PAGE and transferred to Immobilon-P membranes (Millipore). The following primary antibodies were used: anti-p53 (1 : 1000, Cell Signaling), anti-PhosphoSer15 p53 (1 : 1000, Cell Signaling), anti-p21 (C19, 1 : 250, Santa Cruz), anti-pRb (G3-245, 1 : 250, BD Pharmingen), anti-Mcm6 monoclonal antibody (1 : 5, kindly provided by Dr H-J Heidebrecht), anticyclin A (C19, 1 : 500, Santa Cruz), anti-PML (PG-M3, 1 : 200, Santa Cruz) and anti-α-tubulin (B-5-1-2, 1 : 5000, Sigma). Signals were revealed after incubation with anti-mouse or anti-rabbit secondary antibodies coupled to peroxidase (Amersham, UK) by using enhanced chemiluminescence (ECL, Amersham, UK), or SuperSignal (West Pico, Pierce, USA).

Fluorescence microscopy

For fluorescence microscopy, 2×10^5 cells were plated on coverslips positioned on 6-well plates (Costar, Corning Inc., NY, USA). At 24 h after plating, the cells were fixed with 4% paraformaldehyde for 15 min at room temperature. Then, the cells were washed in PBS and permeabilized using ice-cold 0.2% Triton X-100 in PBS/BSA 3% solution for 5 min. Then, the cells were washed three times with PBS/BSA and incubated for 1 h at room temperature with the following primary antibodies: anti-p53 mix (1801, 1 : 100 and 421, 1 : 100, Santa Cruz), anti-Rb (Rb1C1, kindly provided by G Klein, 1 : 100), anti-PML (PG-M3, 1 : 400, Santa Cruz) and anti-PML rabbit polyclonal (kindly provided by KS Chang, 1 : 100). The cells were washed and incubated for 1 h with Oregon-Green and Texas-Red conjugated secondary antibody (1 : 1000, Molecular Probes, Eugene, OR, USA). Finally, the cells were washed three times with PBS, incubated in 300 nM DAPI for 10 min and mounted on microscope slides. Images were obtained using a Nikon Eclipse TE2000-U microscope and the software MetaMorph (Universal Imaging Corp.). The figures were composed with Adobe Photoshop 6.0 and Canvas 8.

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