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Cellular Senescence Induced by p53-*ras* Cooperation Is Independent of p21^{waf1} in Murine Embryo Fibroblasts

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Abstract Oncogenic activation in primary murine fibroblasts initiates a senescence-like cell cycle arrest that depends on the p53 tumor suppressor pathway. Conditional p53 activation efficiently induced a reversible cell cycle arrest but was unable to induce features of senescence. In contrast, coexpression of oncogenic ras with p53 produced an irreversible cell cycle arrest that displayed features of cellular senescence. Introduction of a conditional murine p53 allele (p53val135) into double p53/p21-null mouse embryonic fibroblasts showed that p21waf1 was not required for this effect, since p53−/−;p21−/− double-null cells undergo terminal growth arrest with features of senescence following coexpression of oncogenic Ras and p53. Our results indicate that oncogenic activation of the Ras pathway in murine fibroblasts converts p53 into a senescence inducer through a p21waf1-independent mechanism. J. Cell. Biochem. 92: 514–524, 2004.

Key words: cellular senescence; ras; p53; p21waf1; cell cycle

Ras can transform established immortal but not primary mammalian cells. In primary cells, the ectopic expression of the ras oncogene leads instead to a senescence-like state [Serrano et al., 1997]. Experiments using alleles of ras unable to activate one or another effector pathway of Ras signaling have identified the MAP kinase signal as critical for ras-induced senescence [Lin et al., 1998]. Expression of other oncoproteins located downstream of Ras in this effector pathway, such as Raf or MEK, also induce growth arrest [de Stanchina et al., 1998; Lin et al., 1998].

Oncogenic activation of the Ras pathway in murine fibroblasts initiates a permanent cell cycle arrest that depends on functional p53 and is phenotypically similar to replicative senescence [Serrano et al., 1997; Lin et al., 1998; Ferbeyre et al., 2002] which is the cellular senescence reached by increasing population doublings in primary somatic cells. The signalling from aberrant Ras activity to p53 is not fully understood, and the available data support a simple linear model from oncogenic Ras to p53 via induction of p19ARF [Lin et al., 1998; Palmero et al., 1998; Lin and Lowe, 2001; Ferbeyre et al., 2002]. p19ARF links oncogene activation to the p53 tumor suppressor pathway by inhibiting the Mdm2-dependent degradation of p53 [Sherr and Weber, 2000; Mayo and Donner, 2002]. However, activation of the ARF/p53 pathway results in apoptosis or senescence, depending on the type of oncogenic stress [Sionov and Haupt, 1999], implying that additional signals can modulate the outcome of p53 activation [Ferbeyre et al., 2002].

The tumor suppressor p53 coordinates the cellular response to different kinds of stress [Waga et al., 1994]. The stimuli that regulate p53 activity include DNA damage, altered ribonucleotide pools, hypoxia, oncogenes, cell adhesion, mitotic spindle defects, and redox stress [for a review see Vousden and Lu, 2002; Oren, 2003]. These stimuli are believed to activate p53 by increasing its stability and/or inducing posttranslational modifications that enhance the ability of p53 to act as a transcrip-

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tion factor [Ginsberg et al., 1991; Kern et al., 1991]. The outcome of p53 activity can be reversible cell cycle arrest [Michalovitz et al., 1990; Agarwal et al., 1995] or permanent withdrawal from cell proliferation following the induction of apoptosis or cellular senescence [Lowe and Ruley, 1993; Serrano et al., 1997; Ferbeyre et al., 2002].

The p53 transcriptional target $p21^{waf1/cip1}$ (p21) is a universal inhibitor of CDK–cyclin complex formation [Xiong et al., 1993]. It has also been reported that p21 also binds to and inactivates E2F and PCNA [Waga et al., 1994; Afshari et al., 1996], trancription factors required for G1/S progression. Thus, p21 is a potent inhibitor of cell proliferation. The transcriptional activation of p21waf1 has been shown to play a major role in inducing p53-dependent G1 cell cycle arrest following DNA damage [El-Deiry et al., 1993; Waldman et al., 1995]. However, experiments using fibroblasts derived from p21*waf1* nullizygous mice (p21−/−) show that p21 is not entirely responsible for the p53-induced arrest [Deng et al., 1995], indicating that other p53 targets might be involved [Fiscella et al., 1997; Hermeking et al., 1997]. It has been hypothesized that p21 is an important effector of growth arrest during senescence [Noda et al., 1994; Tahara et al., 1995; Afshari et al., 1996; Bond et al., 1996; Brown et al., 1997; Fang et al., 1999; Stein et al., 1999]. Levels of p21 increase with ageing of primary mammalian cells [Noda et al., 1994; Stein et al., 1999] and this increase occur in both a p53-dependent and -independent manner [Tahara et al., 1995]. Overexpression of p21 in immortal cells induced growth arrest with phenotypic features of senescence [Vogt et al., 1998]. Furthermore, disruption of p21 in diploid human fibroblasts by two sequential rounds of targeted homologous recombination is sufficient to bypass G1 arrest in senescence allowing extended lifespan, but not immortalisation [Brown et al., 1997]. However, in mouse embryo fibroblasts absence of p21WAF1 does not overcome senescence [Pantoja and Serrano, 1999].

Other stimuli able to induce terminal G1 arrest with senescent associated features also activate p21, correlating this increase with the phenotypic effect observed [Xiao et al., 1997; Fang et al., 1999; Chang et al., 1999a,b, 2002; Chen, 2000; Lodygin et al., 2002]. These results lead to the hypothesis that the increase of p21 levels under stress might play an important role on cellular senescence.

To address whether p21 CDK inhibitor plays a role in p53-*ras* cooperation to induce cellular senescence, we took advantage of the mouse temperature-sensitive p53 allele (p53val135) [Michalovitz et al., 1990] that allows conditional and reversible activation of p53. We report that enforced p53 expression in double p53−/−; p21−/− knockout mouse embryonic fibroblasts (MEFs) is not sufficient to induce a permanent cell cycle arrest. We also found that oncogenic activation by ras changes the outcome of p53 activity, promoting a permanent cell cycle arrest with the characteristics of cellular senescence in the absence of p21waf1.

EXPERIMENTAL PROCEDURES

Cell Culture

Primary MEFs from p53−/− mice and p53−/−;p21−/− double-knockout mice, were derived from day 13.5 embryos as described previously elsewhere. Cells expressing murine p53val135 were generated by retrovirus-mediated gene transfer of p53val135 into p53−/− MEFs (p53−/−;ts), p53−/−;p21−/− MEFs (p53−/−;p21−/−;ts cells). Cells expressing Val12-Ras were generated by retrovirus-mediated gene transfer of pBabepuro-Ras(val12) into p53−/− (p53−/−ras), p53−/−;ts (p53−/−;ts-ras), p53−/−;p21−/−;ts (p53−/−;p21−/−;ts-ras cells). Cells were cultured in Dulbecco's modified Eagle medium (GIBCO) supplemented with 10% fetal bovine serum (Sigma), 1% penicillin G–streptomycin sulfate (Sigma).

Retroviral Vectors and Gene Transfer

The following retroviral vectors were used: p53val135 mutant cDNA in pWZLHygro, the pBabe vector and its derivatives with oncogenic ras (H-RasV12). Retrovirus-mediated gene transfer was performed as previously described [Carnero et al., 2000]. Briefly, 5×10^6 LinXE retrovirus producer cells were plated in a 10 cm dish, incubated for 24 h, and then transfected by calcium phosphate precipitation with 20 μg of retroviral plasmid (16 h at 37°C). After 48 h, the virus-containing supernatant was filtered (0.45 μm filter, Millipore, Billerica, MA) and supplemented with 8 μg/ml polybrene (Sigma) and equal volume of fresh media. Prior to infection, 8×10^5 target fibroblasts were plated at cells per 10 cm dish and incubated overnight.

For infections, the culture medium was replaced by the appropriate viral supernatant, then the culture plates were centrifuged (1 h, 1,500 rpm) and incubated at 37°C for 16 h. Infected cell populations were selected in puromycin (2.5 μg/ml, 3 days) for pBabe-derived vectors or in hygromycin (50 μg/ml) for WZL-Hygro-based vectors.

Temperature Shifts and Cell Proliferation Analysis

For the temperature shift experiment, 5×10^5 cells were plated in 10-cm-diameter plates. Cells were grown at 39°C (i.e., never incubated at 32°C) or arrested for the indicated times at 32°C.

For the growth curves: cells were infected as before. At PD 12, 3×10^3 cells were plated in 2.5 cm dishes. At 2–3 day intervals, cells were fixed and stained with crystal violet. After extensive washing, crystal violet was resolubilised in 10% acetic acid and quantified at 595 nm as a relative measure of cell number.

For colony-forming ability assays, cells were plated at 1,000 cells per 10 cm-diameter plate. After 10 days, colonies were stained with crystal violet and counted.

SA β-Gal Activity

Senescence-associated (SA) β-galactosidase (β-Gal) activity was measured as previously described [Dimri et al., 1995], except that cells were incubated in 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) at pH 5.5 to increase the sensitivity of the assay in MEFs. The percentage of cells expressing SA β-Gal was quantified by inspecting 200 cells per 10-cm-diameter plate three times.

Northern Blot

Total RNA was extracted by using RNAzolB (Invitrogen, CA). Samples (10 μg) of total RNA were loaded in formaldehyde-agarose gels and transferred to Hybond membranes (Amersham, Piscataway, NJ). Blots were hybridized with ^{32}P-labeled probes specific for mouse PAI-1 cDNA [Serrano et al., 1997]. A probe specific for 18S rRNA was used to confirm that the same amount of RNA was present in each lane.

Western Blot

Cells were washed twice with ice-cold PBS and lysed in NP-40 lysis buffer (150 mM NaCl, 1% NP-40, 50 mM Tris-HCl pH 8.0, 1 mM PMSF, 1 μg/ml Leupeptin, 25 μg/ml Aprotinin, 1 mM EDTA). After 15 min on ice, samples were vortexed (5 min at 4°) and cleared by centrifugation. Proteins were separated on SDS–PAGE, transferred to Immobilon P membranes (Millipore), probed using anti-p16 antibody (from Santa Cruz, CA), and was detected using biotinilated secondary antibodies (Amersham) followed by HRP-conjugated streptavidin and ECL.

FACs

Cells were cultured under normal conditions at 39°C. The cells were split and plated and 24 h later the medium changed. One day later cells were tripsinized and fixed with 70% methanol. Cells were washed twice with PBS plus 0.1% FCS and incubated in PBS suplemented with RNAse (10 mg/ml) and propidium iodine (5 mg/ml) for 30 min. Then, DNA content was analyzed by FAC Scan.

RESULTS

Using a retroviral vector that expresses temperature-sensitive p53 (p53val135) to conditionally express p53 in p53−/− MEFs we established cell lines from the infected population and called it p53−/−;ts cell line. Similarly, we expressed the temperature-sensitive p53 (p53val135) in the double p53−/−;p21−/− knock-out MEFs (p53−/−;p21−/−;ts). These cell lines were further infected with a retroviral vector carrying oncogenic ras (Val12-ras) generating p53−/−;ts-ras and p53−/−;p21−/−;ts-ras cell lines respectively (Table I). These cell

TABLE I. Summary of Cell Lines Used in This Study

Cell line	Genotype	Phenotype
p53−/−	p53−/−MEFs	Growth at 32 and 39°C
p53−/−; ras	p53−/−MEFs with ras^{val12}	Growth at 32 and 39°C
p53−/−;ts	p53−/−MEFs with $p53^{val135}$	Growth at 39°C, reversible arrest at 32°C
p53−/−;p21−/−;ts	p53−/−;p21−/− MEFs with $p53^{val135}$	Growth at 39°C, reversible arrest at 32°C
p53−/−;ts-ras	p53−/−MEFs with $p53^{val135}$ and ras^{val12}	Growth at 39°C, terminal arrest at 32°C
p53−/−;p21−/−;ts-ras	p53−/−;p21−/− MEFs with $p53^{val135}$ and ras^{val12}	Growth at 39°C, terminal arrest at 32°C

lines were established and maintained at the restrictive temperature (39°C) to avoid selection against p53 or components of the p53 pathway. As control cell lines we infected parental p53 null cells with oncogenic ras (p53−/−;ras) and maintained in the same conditions as before.

To study the effect of the activation of p53 in these cell lines, we examined proliferation at the restrictive (39°C) and permissive (32°C) temperatures. Cells expressing the temperature sensitive mutant of p53 (p53−/−;ts, p53−/−;ts-ras, p53−/−;p21−/−;ts, and p53−/−;p21−/−;ts-ras) grew at 39°C and underwent a cell cycle arrest when shifted to 32°C (Fig. 1). This cell cycle arrest at 32°C was more efficient in the presence of oncogenic ras. In contrast, the parental p53−/− MEFs (lacking p53val135) or expressing ras (p53−/−;ras) did not arrest at 32°C, indicating that the cell cycle arrest we observed was not a temperature effect (Fig. 1).

Oncogenic ras changes the ability of p53 from inducing reversible cell cycle arrest to induce permanent cell cycle arrest with features of senescence [Ferbeyre et al., 2002]. We reasoned that since p21 seems to play a role in cellular senescence and oncogenic ras signalling activates also p21 in primary cells probably through an increase of p53 activity, the presence or absence of p21 might be influencing the outcome of ras and p53 cooperation in oncogene-induced senescence. To examine this possibility, we examined different senescence markers at the restrictive and permissive temperatures. All infected cell populations were initially grown at 39°C, shifted to 32°C for various times and then senescence markers analyzed.

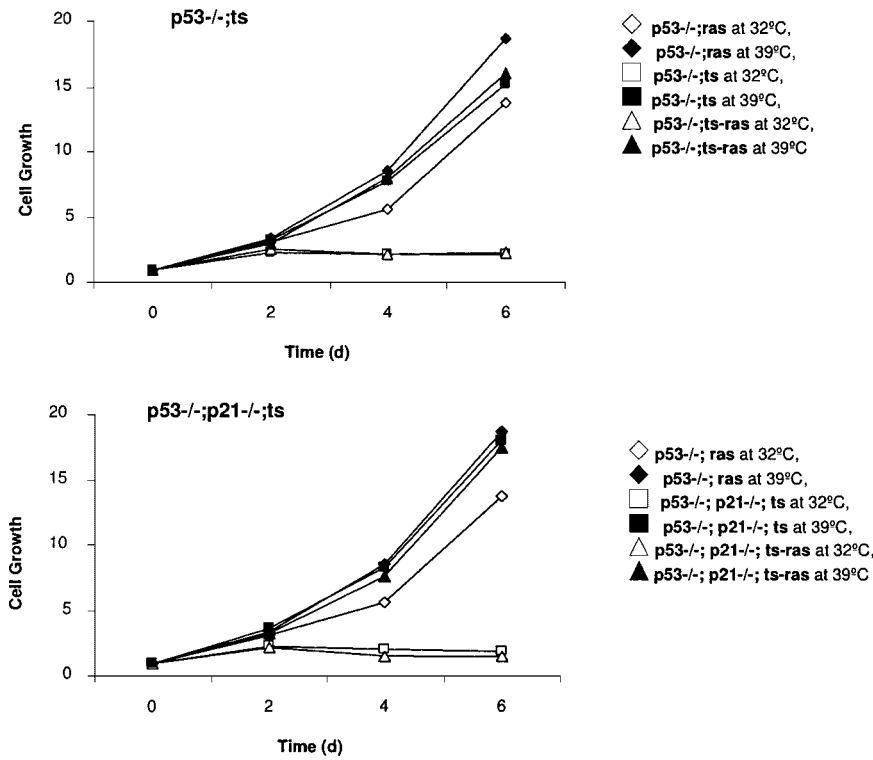

Fig. 1. Growth arrest induced by p53(val135) mutant is independent of the presence of p21 and oncogenic Ras. **Upper panel**: p53−/−;ts cells expressing or not val12-Ras (see text for details) were grown at restricted temperature (39°C; filled symbols), or permissive temperature (32°C; open symbols) for different days. Cells were seeded at 39°C and after 12–14 h shifted to 32°C (day 0). At indicates times, cells were fixed and growth measured as indicated in Materials and Methods. Growth of cells was referred to cells at day 0. (◇) p53−/−;ras at 32°C, (◆) p53−/−;ras at 39°C, (□) p53−/−;ts at 32°C, (■) p53−/−;ts at 39°C, (△) p53−/−;ts-ras at 32°C, (▲) p53−/−;ts-ras at 39°C. **Lower panel**: p53−/−;p21−/−;ts cells expressing or not val12-Ras (see text for details) were grown at restricted temperature (39°C; filled symbols), or permissive temperature (32°C; open symbols) for different days. Cells were seeded at 39°C and after 12–14 h shifted to 32°C (day 0). At indicated times cells were fixed and growth measured as indicated in Materials and Methods. Growth of cells was referred to cells at day 0. (◇) p53−/−;ras at 32°C, (◆) p53−/−;ras at 39°C, (□) p53−/−;p21−/−;ts at 32°C, (■) p53−/−;p21−/−;ts at 39°C, (△) p53−/−;p21−/−;ts-ras at 32°C, (▲) p53−/−;p21−/−;ts-ras at 39°C Data are presented as the average of one representative experiment performed in triplicate. Experiments were repeated three times with similar results.

p53−/−;ts cells incubated at 32°C for 2 or more days did not display any phenotypic senescence markers [Ferbeyre et al., 2002, Fig. 2B], even though they were clearly arrested (Fig. 1A). Similarly, p53−/−;ts-ras cells maintained in the absence of functional p53 at 39°C did not display features of senescence (Fig. 2A). However, p53−/−;ts-ras cells incubated at 32°C for more than 2 days displayed an enlarged and flat morphology (Fig. 2A), This phenotype was indistinguishable from the senescence-like arrest observed in double null p53−/−;p21−/− cells expressing oncogenic ras: p53−/−;p21−/−;ts-ras cells (Fig. 2A).

To confirm these data, we analyzed the percentage of cells that shown senescence associated β-gal activity. p53−/−;ts and p53−/−;p21−/−;ts shown a small percentage of cells (around 10%) with SA-βgal activity either at 32 or 39°C. p53−/−;ts-ras or p53−/−;p21−/−;ts-ras show similar background at restrictive temperature, however, when shifted to 32°C for more than 2 days accumulate cells with senescent associated β-gal activity up to 80% after 4 days (Fig. 2B).

A further confirmation that the phenotype observed was associated to senescence was obtained from the analysis of the expression of PAI-1 mRNA. This has been shown to be specific senescence-associated marker [Serrano et al., 1997]. After 2 days at 32°C, p53−/−;ts and p53−/−;p21−/−;ts cells do not show expression

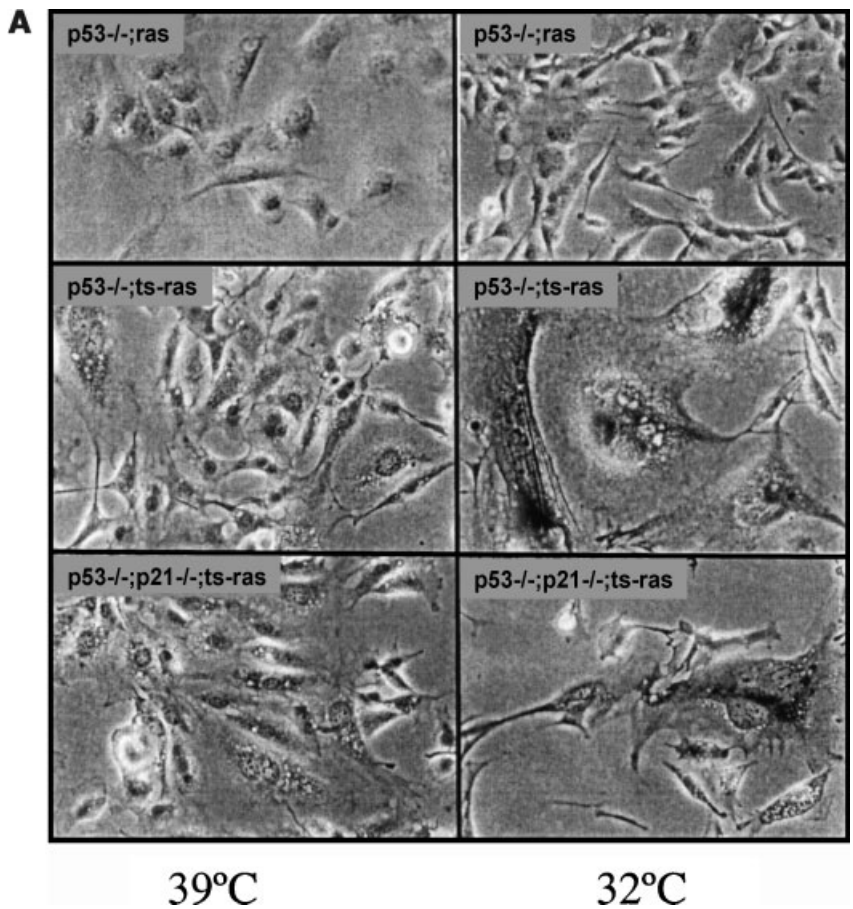

Fig. 2. Oncogenic activation of the Ras pathway during p53-dependent cell cycle arrest induces senescent features in the absence of p21. **A**: Picture of p53−/− cells expressing the thermosensible mutant of p53 (val135) and oncogenic ras (val12) growing at 39°C or arrested at 32°C. p53−/−;ras cells do not express the p53(val135) mutant and were used as control. **B**: Percentage of cells showing senescent-associated β-gal staining in the different cultures after 2, 3, or 4 days at 32°C. **Left panel**: (□) p53−/−;ts at 32°C, (■) p53−/−;ts-ras at 32°C, (△) p53−/−;ts at 39°C, (▲) p53−/−;ts-ras at 39°C. **Right panel**: (□) p53−/−;p21−/−;ts at 32°C, (■) p53−/−;p21−/−;ts-ras at 32°C, (△) p53−/−;p21−/−;ts at 39°C, (▲) p53−/−;p21−/−;ts-ras at 39°C. Data are presented as the average of one representative experiment performed in triplicate. Experiments were repeated three times with similar results. **C**: Northern blot of PAI-1 transcript in the cells after different times incubated at 32°C. Cells were processed as above and mRNA extracted and hybridized as indicated in Materials and Methods. As control we show the ethidium bromide stained 28S band of the total RNA. The experiment was repeated twice with similar results.

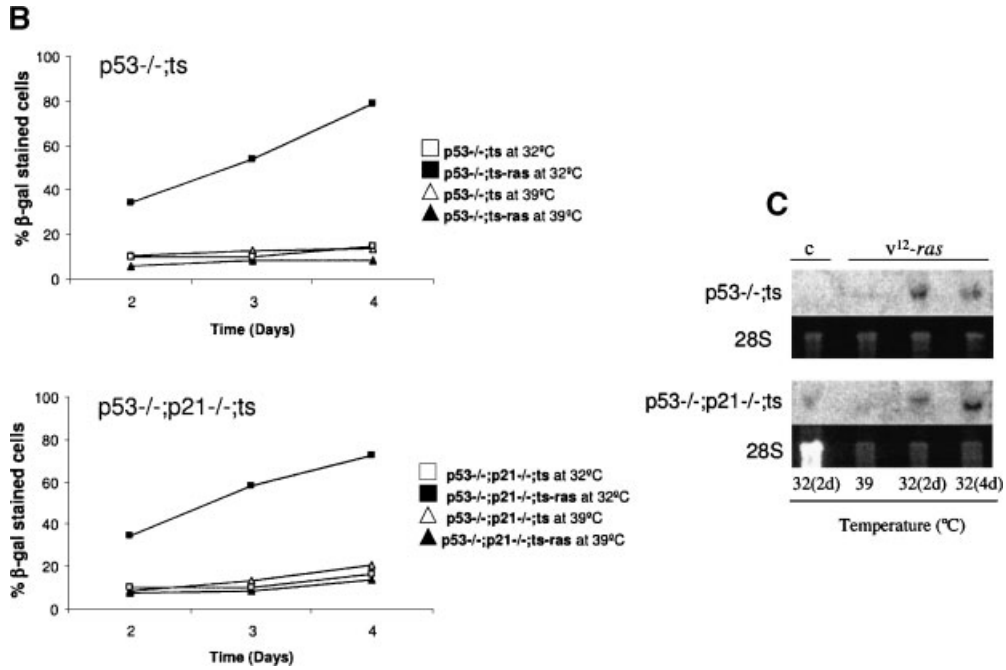

Fig. 2. (*Continued*)

of this marker, indicating that p53-induced arrest is a different phenotype from senescent arrest (Fig. 2C). However, p53−/−;ts-ras and p53−/−;p21−/−;ts-ras cells shifted at 32°C express PAI1 mRNA (Fig. 2C), confirming the specificity of the phenotype elicited by p53 and ras cooperation. These data suggest that p21 is not required in the p53-ras cooperation to induce cellular senescence.

It has been reported that in the presence of oncogenic ras, p53-induced growth arrest is irreversible even if the population is shifted to restrictive temperature again [Ferbeyre et al., 2002]. To study whether p21 has a role in this irreversible arrest, all cell populations were initially grown at 39°C, shifted to 32°C for various times (2 or 4 days), and then returned to 39°C. Cell proliferation was monitored after a return to 39°C (day 0).

The rates of p53−/−;ts and p53−/−;ts-ras growing at 39°C and never incubated at 32°C (day 0) were similar (Fig. 1A). Incubation of these cells at 32°C led to a cell cycle arrest (Fig. 1A). Incubation of p53−/−;ts-ras cells for 2 or 4 days at 32°C dramatically decreased their ability to restore growth after a return to 39°C compared to the p53−/−;ts control (Fig. 3). These data agree with the previously reported studies [Ferbeyre et al., 2002], except that our cultures are unable to re-enter proliferation after a shorter period of time at permissive temperature (2 days). To study the relevance of p21 in this terminal arrest, we carry-out the same recovery protocol with the p21 null cultures: p53−/−;p21−/−;ts and p53−/−;p21−/−;ts-ras cell lines. p53−/−;p21−/−;ts-ras cells did not recover growth after 2 days cultured at 32°C, while the population of cells not expressing oncogenic ras did re-enter cell cycle once shifted to restrictive temperature (Fig. 3). The absence of p21 did not alter the capability of re-entering the cell cycle upon a return to 39°C.

To assess the long-term growth potential of the cells upon a shift from 32 to 39°C, we used a clonogenic assay. This assay measures the proliferation capacity of a cell based on its ability to form colonies after plating at low density. One thousand cells were plated in 10-cm-diameter plates and kept at 39°C for 24 h, then shifted to 32°C for 2 days. Cells were shifted back to 39°C and allowed to grow for 10 days. The plating efficiency of p53−/−;ts and p53−/−;p21−/−;ts cells at 39°C (i.e., never incubated at 32°C) was around 20% and was not altered by expression of ras. In contrast, p53−/−;ts-ras and p53−/−;p21−/−;ts-ras cells lost the ability to form colonies when incubated for more than 2 days at 32°C (Fig. 4). Cells plated at clonogenic density at 32°C never formed colonies (data not shown). Importantly, the differences in clonogenic

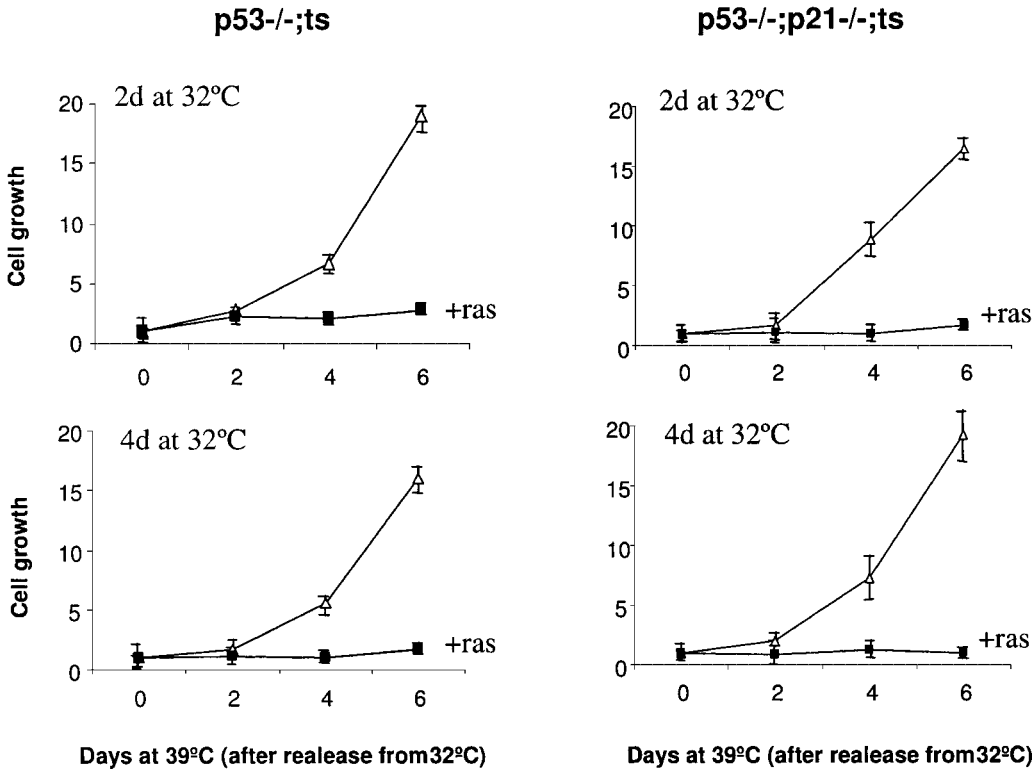

Fig. 3. p21 is not essential in the terminal growth arrest induced by p53-ras cooperation. Growth curves evaluating the effect of the inactivation of p53 after p53-mediated cell cycle arrest. MEFs expressing p53val135 can be grown at 39°C and arrested at 32°C. The arrested populations are then shifted back to 39°C to inactivate p53val135 and investigate their recovery from a cell cycle arrest. **Left panel**: (△) p53−/−;ts, (■) p53−/−;ts-ras. **Right panel**: (△) p53−/−;p21−/−;ts, (■) p53−/−;p21−/−;ts-ras. Shown is the relative amount of cells growing at 39°C for different times after being arrested at 32°C for 2 or 4 days (day 0; moment when cultures were shifted back to 39°C). All values are compared to the value scored at day 0, which is taken as 1. Data are presented as the average of one representative experiment performed in triplicate. Experiments were repeated three times with similar results.

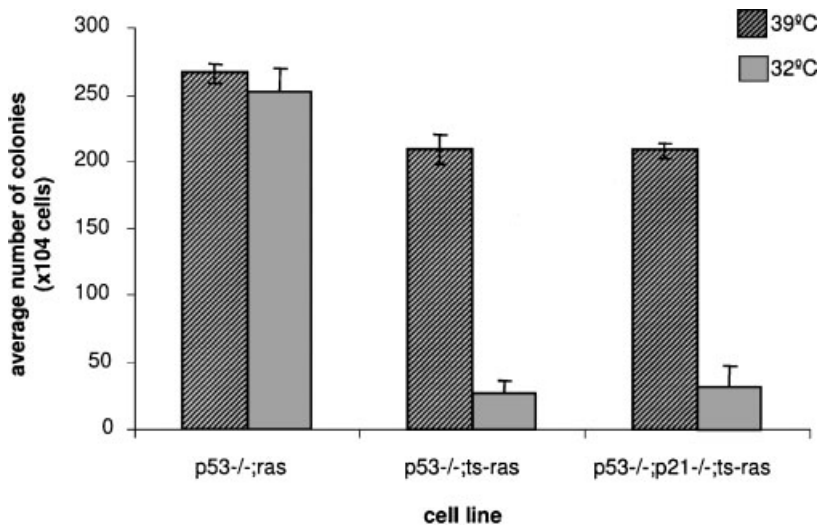

Fig. 4. Colony formation efficiency evaluating the long term effect of the inactivation of p53 after p53-mediated cell cycle arrest. MEFs expressing p53val135 can be grown at 39°C and arrested at 32°C. The arrested populations are then shifted back to 39°C to inactivate p53val135 and investigate their recovery from a cell cycle arrest. One thousand cells were plated in 10-cm-diameter plates and kept at 39°C for 24 h, then shifted to 32°C for 2 days. Cells were shifted back to 39°C and allowed to grow for 10 days.

TABLE II. Cell Cycle Arrest Induced by p53 in Absence of p21

	39°C			32°C		
Cell line	G1	S	G2/M	G1	S	G2/M
p53−/−;ts	38	50	12	56	30	14
p53−/−;p21−/−;ts	44	44	12	58	29	13
p53−/−;ts-ras	41	45	14	67	16	17
p53−/−;p21−/−;ts-ras	48	41	11	63	26	10

potential between these cell populations were not due to a differential ability to adhere to plates at low density since we counted similar numbers of attached cells 24 h after plating (data not shown). We concluded that oncogenic ras transmit signals that impair recovery from a p53-mediated cell cycle arrest, and that this property is independent of the presence of p21.

Finally, we have studied whether the arrest induced by ras-p53 cooperation is similar in the presence or absence of p21. Cell cycle analysis of the arrest induced by replicative senescence in primary MEFs did not show differences between p53−/−;ts and p53−/−;p21−/−;ts cells. Both cell lines arrest at permissive temperature (Table II) and BrDu incorporation was not detected 4 days after reaching senescence (not shown). In our p53-ras cooperation cellular system, we observed similar behavior (Table II). p53−/−;ts-ras and p53−/−;p21−/−;ts-ras cells arrest at G1, indicating that p21 is not the determinant factor for the phase of arrest of the cells.

It has been proposed that terminal growth arrest observed in senescence is due to p16ink4a levels, which are maintained during senescence arrest. We have analyzed the role of p16ink4a in our system. Cells were cultured at 39°C and then shifted to 32°C during 24 or 48 h. Then p16Ink4a levels were analyzed (Fig. 5). We

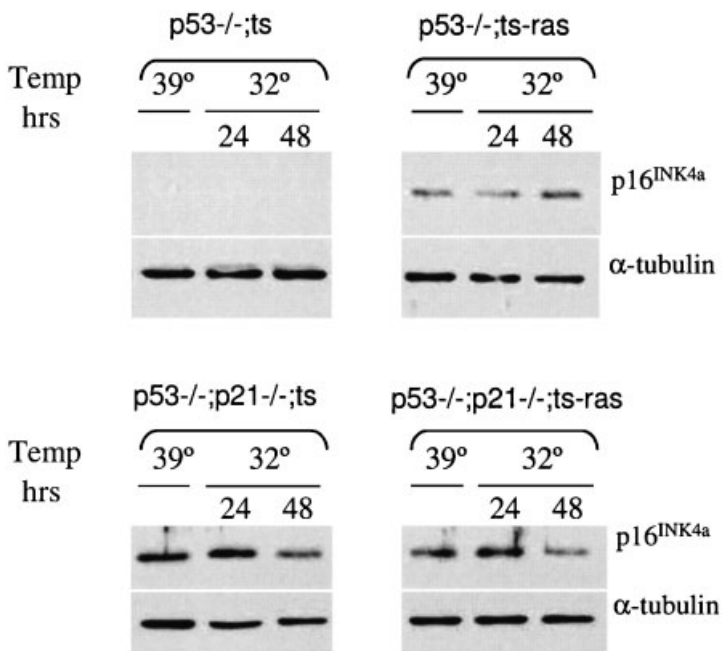

Fig. 5. Levels of p16ink4a do not change after p53-mediated cell cycle arrest or senescence. Different cell lines expressing p53val135, p53−/−;ts, p53−/−;ts-ras, p53−/−;p21−/−;ts and p53−/−;p21−/−;ts-ras were grown at 39°C and switched at 32°C during 24 or 48 h. Then p16INK4a levels were analyzed by immunobloting. The experiment was repeated twice with similar results.

found that in p53−/−;ts cells p16 expression is dependent on the presence of oncogenic ras. However p16 levels do not change upon p53 activation (Fig. 5). In cells null for p53 and p21, p53−/−;p21−/−;ts, p16 levels are high and do not change upon expression of oncogenic ras or activation of p53. These data suggest that p16 is not responsible for senescence-induced arrest by p53 and ras cooperation.

DISCUSSION

Oncogenic ras can activate p53 to promote cellular senescence, which acts to limit the transforming potential of excessive signalling [Serrano et al., 1997; Lin et al., 1998; Pearson et al., 2000; Lin and Lowe, 2001]. Conditional activation of p53 in MEFs produces a reversible cell cycle arrest, whereas activation of p53 in the presence of oncogenic Ras leads to a permanent cell cycle arrest with features of cellular senescence. Despite the observed increase on p21 induced by p53 and observed in Ras induced senescence, the presence of p21 is not relevant in the senescent phenotype elicited by p53-Ras cooperation in MEFs.

The data presented in this study, as well as previous reports, clearly demonstrate that p53 pathway is essential for oncogenic Ras to engage the senescence machinery in murine fibroblasts. We show that oncogenic Ras and p53 cooperate to induce senescence but that after several days of this arrest, a substantial portion of the cells are unable to reenter the cell cycle even when p53 activity has been removed. This observation suggests that p53 is strictly required to initiate a cell cycle arrest but that the maintenance of the senescent state is less dependent on p53. Our data indicate that the presence of p21, a major transcriptional target of p53, is not required for p53-induced arrest in our system and is not essential in the p53-ras cooperation to induce senescence and irreversible growth arrest.

A number of reports indicate that p21 has an essential role in the senescent arrest [reviewed in Carnero et al., 2003], either in the induction of the senescent growth arrest [Noda et al., 1994; Tahara et al., 1995; Ball et al., 1997], due to its ability to inhibit essential activities such as CDK, E2F1, or PCNA [Xiong et al., 1993; Waga et al., 1994; Afshari et al., 1996], or in the maintenance of its terminal arrested phenotype [Vogt et al., 1998; Fang et al., 1999]. The role of p21 has been proposed not only in replicative senescence [Brown et al., 1997; Stein et al., 1999], but also in oncogenic stress-induced senescence, or in chemotherapy-induced senescence [Xiao et al., 1997; Chang et al., 1999a,b, 2002; Chen, 2000; Lodygin et al., 2002]. In agreement with those of Pantoja and Serrano [1999], our data suggest that p21 is not essential in the Ras induced senescence in MEFs, and that other p53-dependent transcripts might be required for this effect. p53 signalling pathway plays a direct role in senescence [Atadja et al., 1995; Bond et al., 1996]. p53 controls the expression of a number of known genes involved in growth arrest, including p21, 14-3-3-σ, GADD45, and WIP1 [Wang et al., 1999; Taylor and Stark, 2001]. p53 induction also results in the repression of a number of genes necessary to sustain cell growth such as myc, fos, jun, DNA polymerase α, RB1, and bcl2 [El-Deiry, 1998]. Our data suggest that neither p21 or p16ink4 are fully responsible for the growth arrest induced by senescence, and other transcriptional targets of p53 and ras must play an active role in this phenotype. In this regard, genes regulating G1 entry (such as repression of myc, fos, jun, DNA polymerase α, or RB1) seems more likely to be responsible for the observed effect.

Thus, senescence could be triggered by p53 through transcriptional activation/repression of specific target genes and cellular immortalisation could be achieved by altering the levels of the p53-dependent transcripts to block the changes occurring at senescence. Some of these changes have been occurring during proliferative-, oncogenic-, or drug-induced senescence [reviewed in Carnero et al., 2003].

However, our data, and those of Ferbeyre et al. [2002] and Pantoja and Serrano [1999], which depict p21 as no essential in any of the senescent states independently of the inducers stimulus, have been done in mouse embryo fibroblasts, while many reports describing an essential role of p21 in replicative senescence [Brown et al., 1997] oncogene induced senescence [Wei et al., 2001] or drug-induced senescence [Chang et al., 1999a,b, 2002; Chen, 2000; Roninson, 2002] have also been described in human cells. This might account for a physiologically relevant role of p21 in human cells but not in mouse cells, despite similarity in biochemical behavior. To discriminate this possibility, more experiments in comparable models are necessary.

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