

Inhibition of p21-mediated ROS accumulation can rescue p21-induced senescence

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The cyclin-dependent kinase (CDK) inhibitor p21^{Waf1/Cip1/Sdi1} was identified initially as a gene induced in senescent cells and itself has been shown to cause permanent growth arrest/senescence. Reactive oxygen species (ROS), a byproduct of oxidative processes, can also induce an irreversible growth arrest similar to senescence. Here we show that p21 increased intracellular levels of ROS both in normal fibroblasts and in p53-negative cancer cells. *N*-acetyl-L-cysteine, an ROS inhibitor, rescued p21-induced senescence, showing that ROS elevation is necessary for induction of the permanent growth arrest phenotype. p16^{Ink4a}, a CDK4- and CDK6-specific inhibitor, failed to increase ROS levels, and cell cycle arrest induced by p16 was reversible following its down-regulation, demonstrating the specificity of this p21 effect. A p21 mutant that lacked the ability to bind proliferating cell nuclear antigen (PCNA) retained the ability to induce both ROS and permanent growth arrest. All of these findings establish that p21 mediates senescence by a mechanism involving ROS accumulation which does not require either its PCNA binding or the CDK inhibitory functions shared with p16.

Keywords: CDK inhibitors/p21/PIG3/ROS/senescence

Introduction

Replicative senescence is a terminal differentiation state in which metabolically active cells are permanently and irreversibly arrested, with distinctive morphological changes (Hayflick and Moorehead, 1961) and phenotypic markers (Dimri *et al.*, 1995). This phenotype was first observed in normal human fibroblasts in culture, which have a finite replicative lifespan and then become permanently growth arrested (Hayflick and Moorehead, 1961). Senescence can also be triggered in normal cells by oncogenes such as Ras (Serrano *et al.*, 1997) or Raf (Zhu *et al.*, 1998) or by tumor suppressors such as p53 (Sugrue *et al.*, 1997). Thus, it has been suggested that senescence acts as a tumor suppressor mechanism by controlling the

emergence of immortal cells (Sager, 1991; Smith and Pereira-Smith, 1996; Serrano *et al.*, 1997).

Reactive oxygen species (ROS), byproducts of normal cellular oxidative processes (Orr and Sohal, 1994), have been shown to be involved in senescence (Q. Chen *et al.*, 1995). Senescent cells are known to have higher levels of ROS than normal cells (Hagen *et al.*, 1997). Moreover, oxidative stress caused by sublethal doses of H₂O₂ (Chen *et al.*, 1998) or hyperoxia (von Zglinicki *et al.*, 1995) can force human fibroblasts to arrest in a senescent-like fashion (Chen and Ames, 1994; Caldini *et al.*, 1998; Dumont *et al.*, 2000). Overexpression of antioxidant genes such as superoxide dismutase or catalase causes extension of lifespan in *Drosophila* (Orr and Sohal, 1994; Parkes *et al.*, 1998). This can also be observed in cell cultures maintained in low oxygen environments (Kinnula and Hassinen, 1981). All of these findings point to a strong relationship between oxidative damage, senescence and aging (for a review see Martin *et al.*, 1996).

Up-regulation of the p53 tumor suppressor gene has been implicated in the induction of permanent growth arrest/senescence or apoptosis depending on many factors, including cellular context (Kastan *et al.*, 1991; Lowe *et al.*, 1993; Polyak *et al.*, 1996; Sugrue *et al.*, 1997; Sionov and Haupt, 1999). p53 overexpression has also been shown to cause the accumulation of ROS, presumably mediated by p53 transcriptional influence on pro-oxidant genes (Polyak *et al.*, 1997). p21^{Waf1/Cip1/Sdi1}, a p53-inducible protein (el-Deiry *et al.*, 1993), is a necessary mediator of p53-induced cell cycle arrest (el-Deiry *et al.*, 1993; Waldman *et al.*, 1995, 1996), as indicated by the fact that p53 cannot induce arrest after DNA damage in p21-null mice (Brugarolas *et al.*, 1995; Deng *et al.*, 1995). p21 is a member of a family of cell cycle inhibitors that includes p27 and p57 (Sherr and Roberts, 1995), and it is capable of inhibiting cyclin-dependent kinases (CDKs) (Harper *et al.*, 1993), key regulators of the cell cycle. It also acts to block DNA replication by binding to proliferating cell nuclear antigen (PCNA) (Waga *et al.*, 1994). p21 expression has been observed in cultured human fibroblasts after prolonged passage, during which such cells undergo senescence (Noda *et al.*, 1994). Moreover, p21 has been shown to be capable of inducing permanent growth arrest/senescence in a p53-independent manner (Fang *et al.*, 1999; Wang *et al.*, 1999). This phenotype was identified by use of a tetracycline (tet)-regulatable p21 system in which senescence could be distinguished from cell cycle inhibition associated with p21 expression by the ability to down-regulate p21 expression (Fang *et al.*, 1999). In view of the implication of ROS in the induction of the permanent growth arrest/senescence phenotype, we investigated the possible role of ROS in p21-induced senescence.

Results

p21 increases ROS levels in EJ cells

Expression of p21 in EJ tumor cells has been shown to cause growth arrest (Fang *et al.*, 1999), which becomes irreversible after four or more days of expression. This permanent growth arrest is accompanied by the appearance of the senescence-specific marker SA- β -gal (Dimri *et al.*, 1995) as well as morphological changes similar to those seen in senescence. In view of the known relationship between ROS accumulation and senescence (Q.Chen *et al.*, 1995, 1998), we investigated whether ROS generation might contribute to this phenotype induced by prolonged p21 expression. To do so, we measured intracellular levels of ROS in EJ cells with tet-regulatable p21 expression (EJp21) by staining the cells with 2',7'-dichlorofluorescein diacetate (DCF), a fluorescent marker of cellular oxidant production (Royall and Ischiropoulos, 1993). Figure 1A shows that ROS accumulation occurred by 2 days of tet removal and increased further by 5 days, when growth arrest had become irreversible. We further confirmed this result by measuring the levels of intracellular glutathione, one of the principal ROS buffers and a marker of oxidative stress (Dolphin *et al.*, 1989). As shown Figure 1B, levels of glutathione decreased steadily after p21 induction, consistent with the effects of increased ROS levels. Moreover, when p21 protein levels were titrated using different concentrations of tet, ROS levels varied proportionally (Figure 1C), indicating that the accumulation of ROS was directly dependent on the levels of p21. Since EJ cells are known to lack functional p53 (Sugrue *et al.*, 1997), these results establish that ROS accumulates as a result of p21 induction, independently of p53.

p21 increases ROS levels in normal human fibroblasts

To assess whether ROS production in response to p21 also occurred in normal cells, we expressed p21 in human normal diploid fibroblasts (501T) by retroviral infection. As shown in Figure 2A and B, p21 caused both growth arrest and increased ROS levels, similar to the response observed in EJp21 cells. Cell size also increased in response to exogenous p21 (Figure 2C), as has been reported in naturally senescent fibroblasts (Angello *et al.*, 1989). The levels of p21 expression induced by retrovirus gene transfer were comparable with those induced in response to UV irradiation, a p53-dependent inducer of p21 (data not shown). Thus, ROS increase and the permanent growth arrest phenotype were also induced by p21 expression in normal human cells.

Blocking of ROS rescues p21-induced senescence in EJ cells

To determine whether accumulation of ROS played a major role in p21-induced permanent growth arrest/senescence, we attempted to block ROS accumulation in EJp21 cells with *N*-acetyl-L-cysteine (NAC), a reduced glutathione (GSH) provider and a direct scavenger of ROS (Staal *et al.*, 1990). As shown in Figure 3A, cells incubated with 10 mM NAC showed no reduction in the level of induced p21 protein and maintained ROS levels comparable to those of uninduced cells. The number of cells that

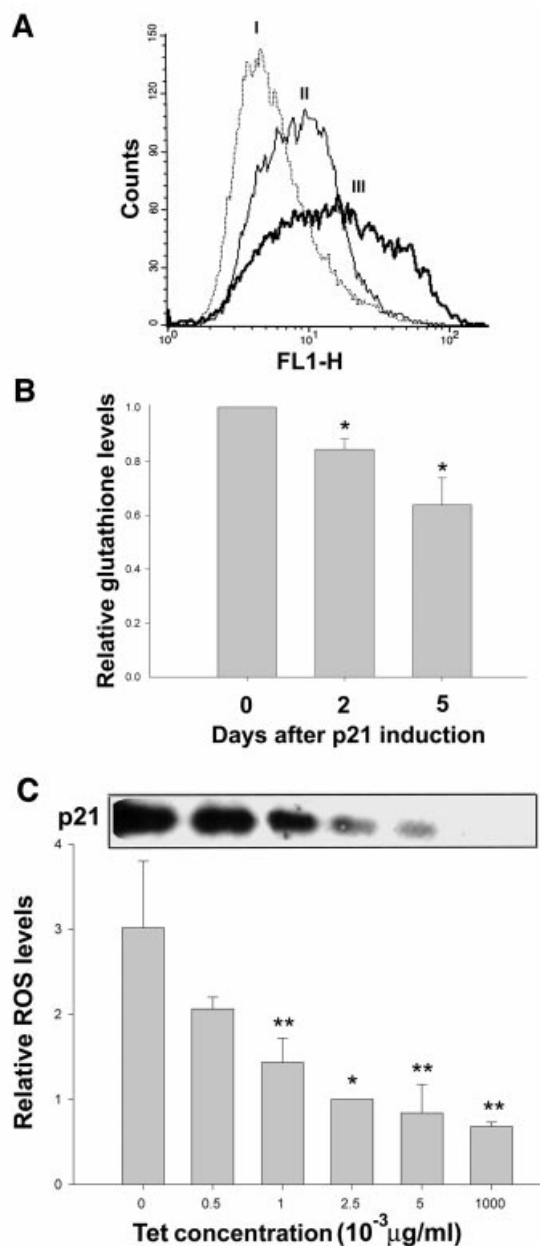


Fig. 1. Changes in ROS levels in EJp21 cells induced by p21 expression. (A) ROS levels as measured by FACS analysis after staining with the fluorescent probe DCF. The curves correspond to (I) cells cultured in the presence of tet (control) and 2 (II) or 5 (III) days after tet removal. The shift to the right of the curve due to increased fluorescence indicates an increase in the intracellular levels of ROS. (B) Intracellular glutathione levels were measured as described in Materials and methods at 2 or 5 days of p21 induction, compared with control cells. Results represent mean values of two experiments, and error bars show the standard deviation. * $P < 0.007$. (C) Immunoblot analysis of p21 expression levels in lysates of EJp21 cells exposed to varying tet concentrations for 3 days. ROS levels in cultures were measured for the same tet concentrations at 3 days. Relative ROS levels were normalized to those at 2.5×10^{-3} μ g/ml. Results represent mean values of three experiments for each concentration, and error bars show the standard deviation. * $P < 0.007$; ** $P < 0.05$.

scored positive for SA- β -gal after 5 days was also substantially reduced in the presence of NAC, as compared with untreated p21-induced cultures (Figure 3B).

Previous studies have shown that with increased time of p21 overexpression, there is a progressive decrease in the

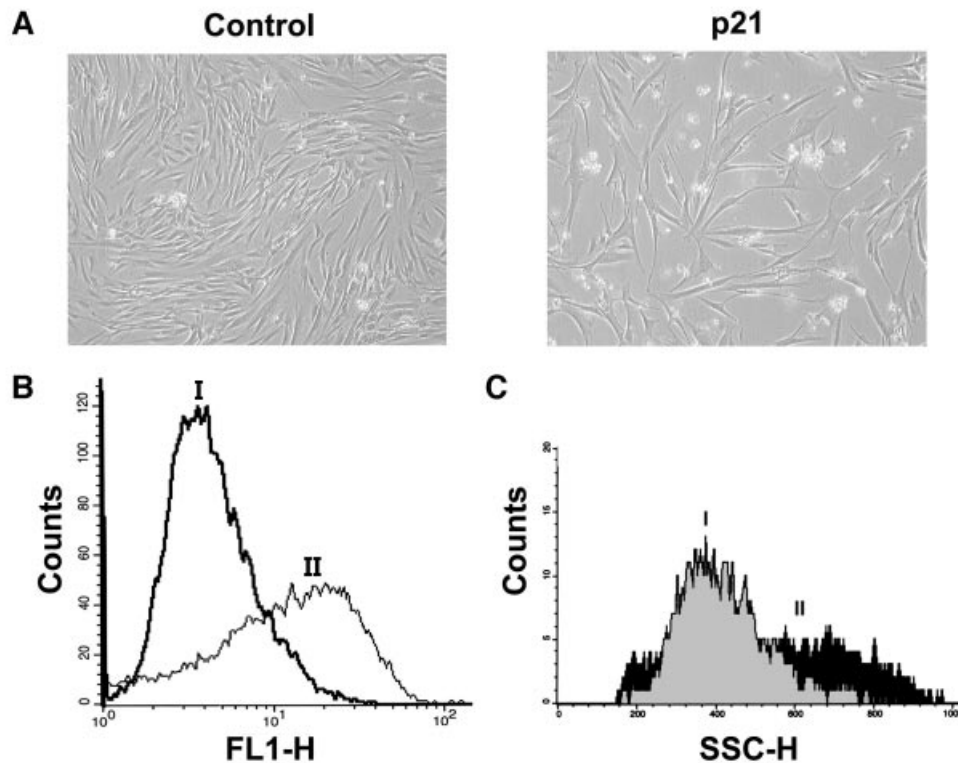


Fig. 2. Increased ROS levels in 501T human fibroblasts in response to p21. (A) 501T cells infected with a retrovirus containing p21 and selected with puromycin for 1 week (p21), showing morphology changes and growth inhibition compared with the vector-infected cells (Control). Cells were photographed using a Nikon Eclipse TE200 microscope (100 \times). (B) ROS levels in cells infected with (I) a retrovirus control vector or (II) containing p21, 1 week after puromycin selection. (C) Cell size of 501T cells as measured by FACS analysis after infection with vector (I) or p21 retrovirus (II).

capacity of cells to form colonies after p21 repression by tet (Fang *et al.*, 1999). To explore the importance of ROS in the irreversible growth arrest/senescence phenotype induced by p21, we examined the effect of exposure to 10 mM NAC on colony formation following p21 repression after p21 overexpression for different time periods. As shown in Figure 3C and D, colony formation of cells treated with NAC during the period of p21 induction was almost comparable to that of uninduced cells, even after 5 days of p21 expression. Figure 3D also shows the results of exposure to 10 mM *N*-acetyl-L-alanine, a compound with chemical structure similar to NAC but with no anti-ROS activity (Qiu *et al.*, 1996), and 2 mM GSH, a widely used ROS inhibitor. Whereas *N*-acetyl-L-alanine failed to protect the cells from the permanent growth arrest phenotype induced by p21, exposure to GSH had an effect comparable with that of NAC. All of these findings indicate that ROS inhibitors were effective in preventing the onset of a permanent growth arrest in response to p21 expression, implicating ROS accumulation in this response.

p16 induces reversible growth arrest without elevation of ROS levels

p16^{Ink4a} is a CDK4 and CDK6 inhibitor that arrests cells in the G₁ phase of the cell cycle (Serrano *et al.*, 1993) and has been reported to be increased in senescent cells (Stein *et al.*, 1999). Moreover, it has been shown that p16 can induce growth arrest when overexpressed in cancer cells (Uhrbom *et al.*, 1997). To determine whether p21-mediated

ROS induction was specific or also resulted from overexpression of other CDK inhibitors, we measured ROS levels in EJ cells, which express no wild-type endogenous p16, containing tet-regulatable p16 (EJp16). As shown in Figure 4A and B, increased p16 levels were accompanied by cell cycle arrest in G₁, together with increased cell size (Figure 4C). It is of note that ROS levels did not increase during p16 induction of growth arrest (Figure 4D). The growth inhibition associated with p16 was reversible following p16 repression (Figure 4E and F). After 5 days of p16 expression, >60% of cells were able to form colonies following tet repression of p16. Under these conditions, NAC did not change the number of rescued colonies significantly. These results indicate that cell cycle inhibition alone cannot account for ROS generation in response to p21 and strengthen the conclusion that ROS contributes importantly to the permanent growth arrest/senescence phenotype observed after p21 expression.

p21's ability to induce ROS and permanent growth arrest is independent of its PCNA-binding function

An important difference between the p21 and p16 families of CDK inhibitors is the ability of the first group to bind to PCNA. To elucidate whether its PCNA-binding function was responsible for the ability of p21 to induce ROS and permanent growth arrest, we established a tet-regulatable EJ cell line expressing a mutant p21 (p21m) that lacks the ability to bind to PCNA (EJp21m). p21m has two mutations, M147A and F150A, which were constructed based on crystal structural analysis that indicates that

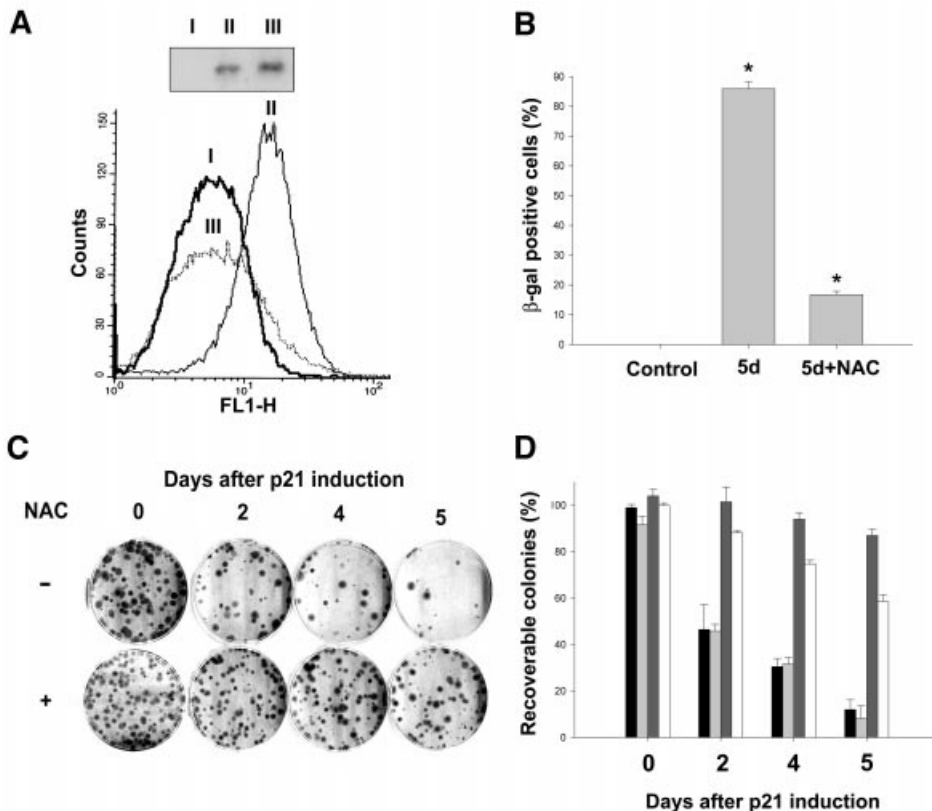


Fig. 3. Effects of NAC on the growth arrest/senescence phenotype of EJp21 cells. (A) ROS levels of EJp21 cells (I) in the presence of tetracycline, (II) after 5 days of p21 induction and (III) after 5 days of p21 induction with the addition of 10 mM NAC. Immunoblot analysis shows p21 expression levels in these cells. (B) The percentage of SA-β-gal-positive cells in EJp21 cells induced to express p21 by tet removal for 5 days in the presence or absence of 10 mM NAC. Control cells were kept in tet and no NAC for the duration of the experiment. Results represent mean values of two independent measures, and error bars show the standard deviation. * $P < 0.0001$. (C) Representative plates from a colony formation assay, in which ~100 cells were plated initially. Cells were maintained in the absence of tet for 0 (Control), 2, 4 or 5 days, and then tet was re-added to the medium. Cells were cultured for 14 more days followed by 10% formalin fixation and Giemsa staining. (D) Percentage of recoverable colonies for each condition described above, normalized to the number of colonies in the control plate. Bars correspond, from left to right, to control plates (black) and plates treated with 10 mM *N*-acetyl-L-alanine (light gray), 10 mM NAC (dark gray) or 2 mM GSH (white). Results represent mean values of at least two experiments, and error bars show the standard deviation.

M147 and F150 are both involved in critical contacts with PCNA (Gibbs *et al.*, 1997). To verify that p21m was deficient in binding to PCNA, transient transfection experiments were carried out followed by hemagglutinin (HA) immunoprecipitation of extracts containing metabolically ^{35}S -labeled proteins. As shown in Figure 5A, HA-tagged wild-type p21 stoichiometrically co-immunoprecipitated a 35 kDa species (lane 1), while p21m was completely deficient in this interaction (lane 2). Immunoblot analysis of the same immunoprecipitates revealed that the 35 kDa species was PCNA (Figure 5B, top panel). Previous studies have revealed the association between endogenous p21, PCNA, CDK and cyclins, using antibodies that recognize CDK or cyclins (Xiong *et al.*, 1992). In the present transient transfection study, HA-p21-PCNA was the predominant complex observed due to the presence of high levels of both proteins. However, consistent with the findings of Xiong *et al.* (1992), the results of immunoprecipitation/immunoblot experiments revealed the presence of CDK2 in both the HA-p21 and HA-p21m immunoprecipitates (Figure 5B, lower panel).

Induction of p21m (Figure 5C) caused the cells to arrest in G_1 (data not shown) in a senescent-like fashion, with expression of SA-β-gal (Figure 5D and E). Moreover, ROS levels increased up to 3-fold by 5 days (Figure 5F), and growth arrest also became irreversible after 4–5 days (Figure 5G). To assess the effects of p21m in normal cells, we infected 501T fibroblasts with a retrovirus containing p21m. As shown in Figure 5H, infected cells became growth arrested and adopted a senescent-like morphology with increased ROS levels similar to those seen in 501T cells infected with a retrovirus containing wild-type p21. These results together show that the ability of p21 to induce permanent growth arrest and ROS in both normal and cancer cells is independent of its PCNA-binding properties.

Further analysis of p21-induced ROS

Recent studies have indicated that p21 can be phosphorylated and translocated to the cytoplasm, where it may exhibit new functions (Zhou *et al.*, 2001). To assess p21 subcellular localization, we performed p21 immunostain-

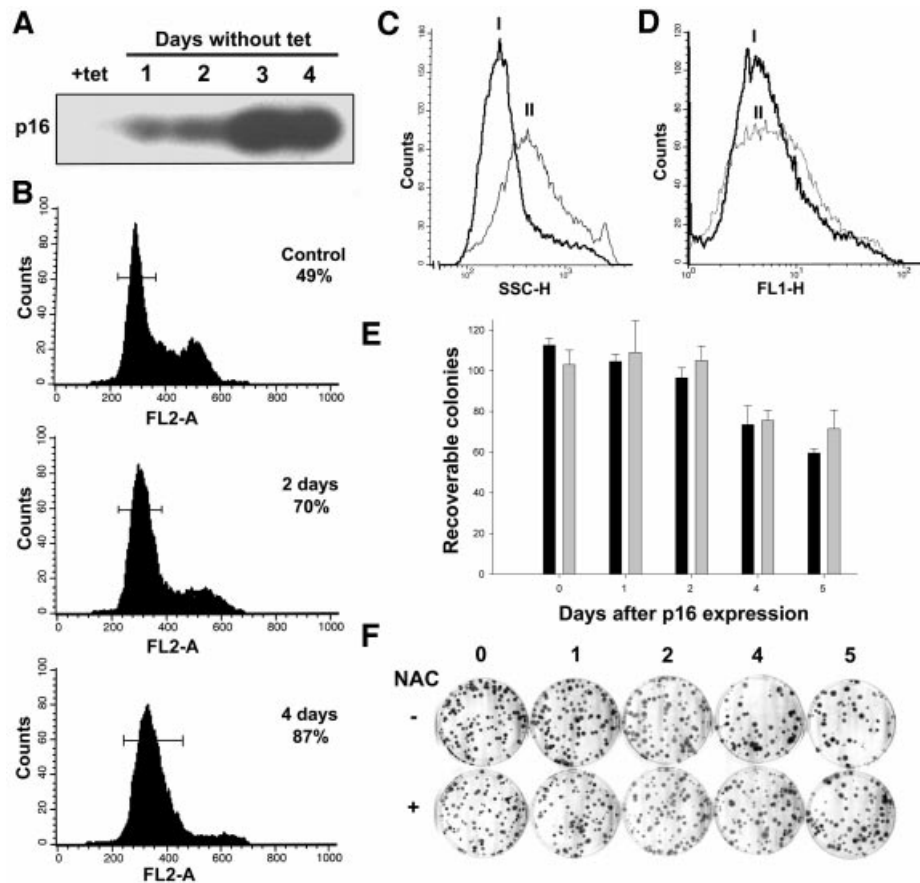


Fig. 4. Effects of p16 induction on the growth arrest/senescent phenotype of EJp16 cells. (A) EJp16 cell lysates obtained at varying time periods following tet removal were subjected to immunoblot analysis with anti-p16 antibody. (B) FACS analysis of EJp16 cells after propidium iodide staining at different times following tet removal. Percentages indicate the number of cells arrested in the G₁ phase of the cell cycle, delimited by the bars in the graphics. (C) Cell size of EJp16 cells (I) cultured in tet or (II) after 6 days of tet removal, as measured by FACS analysis. (D) ROS levels as measured by FACS analysis after staining with the fluorescent probe DCF. The curves correspond to EJp16 cells (I) cultured in the presence of tet or (II) for 6 days following tet removal. (E) Percentage of recoverable colonies following p16 expression for different times. EJp16 cells were plated at a density of 100 cells per plate and were maintained in the absence of tet for 0 (control) up to 5 days, followed by tet addition. Cells were cultured for 14 more days followed by 10% formalin fixation and Giemsa staining. Black bars correspond to results with cultures in the absence of NAC, and gray bars correspond to results with cultures in 10 mM NAC, normalized to the number of colonies in the control plate. Results represent mean values of three experiments, and error bars show the standard deviation. (F) Representative plates from a colony formation assay.

ing following p21 up-regulation in EJp21 cells. As shown in Figure 6A, induced p21 was localized predominantly to the nucleus after both 2 and 5 days. These results indicate that if cytoplasmic localization plays a role in ROS induction, it must be due to a small fraction of the protein.

It has also been reported that p53 increases the levels of intracellular ROS and that this effect is necessary for p53-induced responses, such as apoptosis (Polyak *et al.*, 1997). p53-mediated induction of ROS has been proposed to be dependent on its transactivation of genes capable of generating ROS. *PIG3*, a gene homolog of oxidoreductase genes from several species, is one of the p53-responsive genes that has been suggested to be important in p53 induction of increased ROS levels (Polyak *et al.*, 1997). *PIG3* is induced with delayed kinetics as compared with other p53 target genes and its levels are increased during p53-mediated growth arrest (Flatt *et al.*, 2000). To investigate further the mechanisms of ROS induction by p21, we measured the levels of *PIG3* mRNA in EJp21 cells after tet removal. *PIG3* mRNA levels increased significantly in EJp21 after 5 days of induction (Figure 6B). In

contrast, p16 expression did not increase *PIG3* RNA, consistent with the inability of p16 to induce ROS.

Discussion

Our present studies demonstrate that up-regulation of the CDK inhibitor p21 caused increased ROS levels in both normal and tumor cells, and this increase was proportional to the level of p21. ROS accumulation was not a general consequence of cell cycle arrest, since another CDK inhibitor, p16, failed to increase ROS levels. That ROS accumulation in response to p21 was causative of the permanent cell cycle arrest induced by p21 derives from two lines of evidence. NAC, an inhibitor of ROS (Staal *et al.*, 1990), was able to block ROS accumulation in response to tet-regulatable p21, although it had no effect on the cell cycle inhibition or morphology changes accompanying p21 up-regulation. In striking contrast, NAC inhibited SA- β -gal staining of p21-expressing cells and protected the cells from the permanent growth arrest phenotype induced by p21 expression. Prolonged expres-

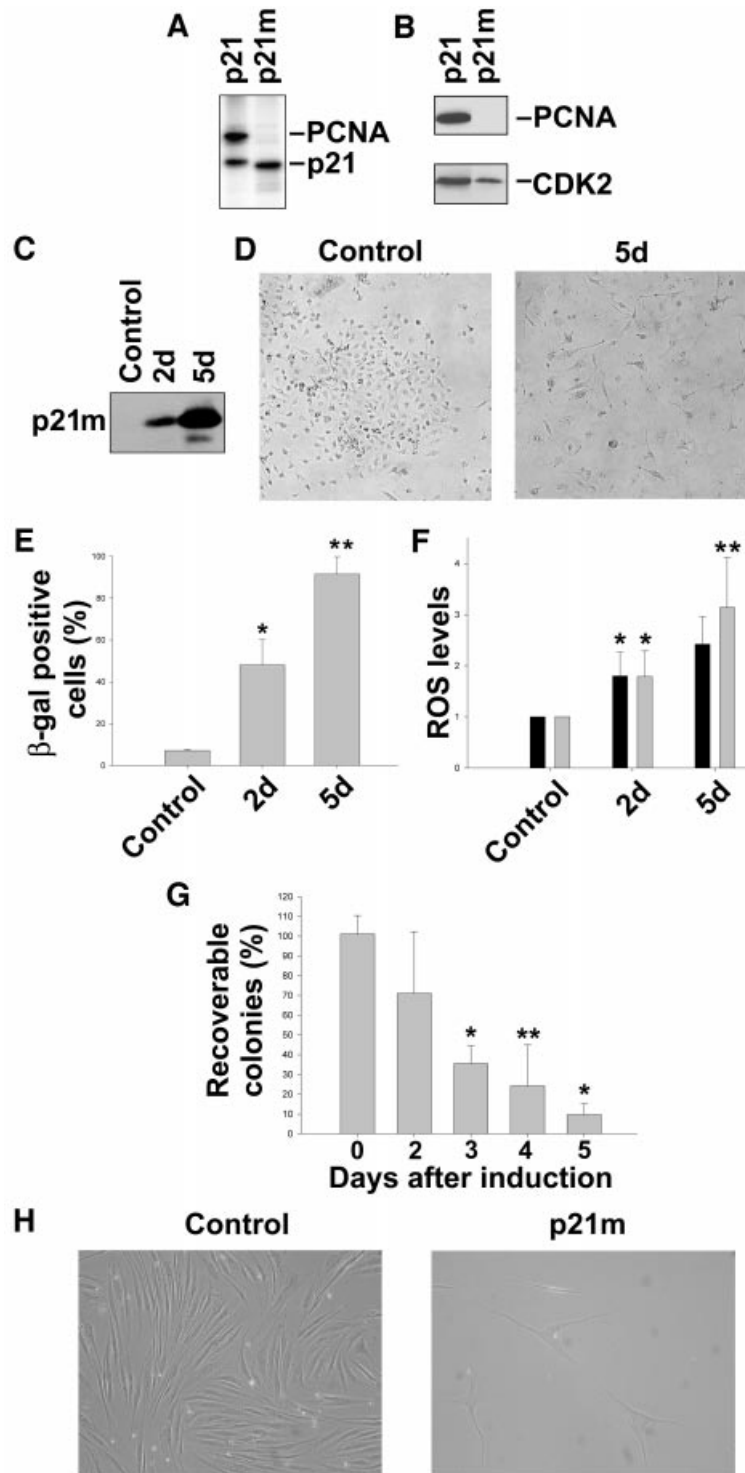


Fig. 5. The mutant form of p21 that does not bind to PCNA induces permanent growth arrest and ROS in EJ and normal human fibroblasts. (A) Autoradiography of a 12.5% SDS-PAGE that separates α HA-immunoprecipitates derived from 35 S-metabolically labeled extracts of 293T cells transfected with HA-p21 or HA-p21m constructs. Polypeptides corresponding in sizes to p21 and PCNA, respectively, are indicated. (B) Immunoblot analysis of PCNA and CDK2 expression levels in the same metabolically labeled extracts. (C) Immunoblot analysis of p21 expression levels in lysates of EJp21m cells after 2 or 5 days of tet removal. (D) EJp21m cells cultured in the presence of tet (Control) or after 5 days of tet removal (5d). Cells were photographed using a Nikon Eclipse TE200 microscope (100 \times). (E) Percentage of SA- β -gal-positive cells in EJp21m cells induced to express mutant p21 by tet removal for 2 (2d) or 5 (5d) days, compared with cells cultured in the presence of tet (Control). Results represent mean values of three independent measures, and error bars show the standard deviation. * $P < 0.05$; ** $P < 0.00001$. (F) ROS levels in EJp21m cells after 2 (2d) or 5 (5d) days of tet removal, compared with levels in EJp21 cells in the same conditions. Control cells were cultured in the presence of tet. * $P < 0.05$; ** $P < 0.001$. (G) Percentage of recoverable colonies following mutant p21 expression for different times. EJp21m cells were plated at a density of 100 cells per plate and were maintained in the absence of tet for 0 (control) up to 5 days, followed by tet addition. Cells were cultured for 14 more days followed by 10% formalin fixation and Giemsa staining. Results represent mean values of three experiments normalized to the number of colonies in the control plate, and error bars show the standard deviation. * $P < 0.00005$; ** $P < 0.0005$. (H) 501T cells infected with a retrovirus containing mutant p21 after 1 week selection with puromycin (p21m), showing morphology changes and growth inhibition compared with the vector-infected cells (Control). Cells were photographed using a Nikon Eclipse TE200 microscope (100 \times).

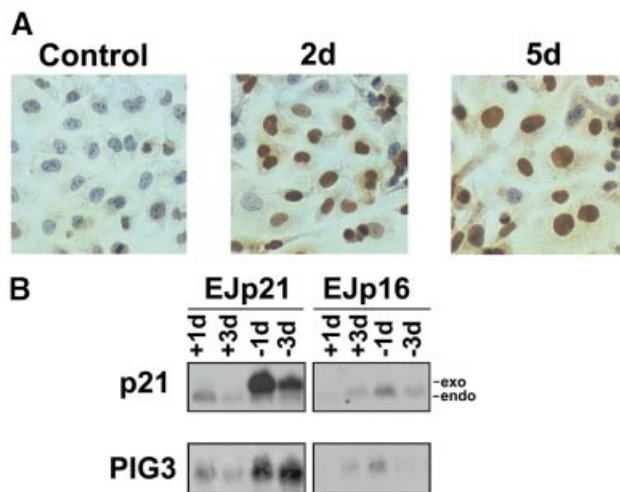


Fig. 6. Further analysis of p21-induced ROS. (A) Immunostaining with p21 antibody of EJp21 cells in the presence of tet for 2 days (Control) and after 2 (2d) or 5 (5d) days of p21 induction by tet removal. Cells were photographed using a Nikon Microphot-FXA microscope (400 \times). (B) PIG3 is induced by p21 or mutant p21 but not p16. Northern blot analysis of p21 and PIG3 RNA levels in EJp21 cells in the presence of tetracycline for 1 or 3 days (+1d and +3d) and after 1 or 3 days of induction (-1d and -3d), compared with levels in EJp16 in the same conditions. Exo, induced p21; endo, endogenous p21.

sion of p16, like p21, can induce senescence-like arrest in cancer cells (Uhrbom *et al.*, 1997). We similarly observed growth arrest in both normal and tumor cells induced by p16. However, growth arrest following p16 down-regulation was found to be reversible, consistent with previous studies that reported reversibility of p16-mediated arrest (Uhrbom *et al.*, 1997; Rossi *et al.*, 1998). It is of note that this reversible growth arrest phenotype correlated with the absence of ROS accumulation in response to p16. All of these findings argue strongly that ROS accumulation is responsible for the permanent growth arrest/senescence phenotype induced by prolonged p21 expression.

It has been shown that naturally senescent fibroblasts exhibit increased ROS (Q.Chen *et al.*, 1995), p16 (Alcorta *et al.*, 1996) and p21 levels (Noda *et al.*, 1994), although p21 up-regulation has been reported to be transient in such cells (Alcorta *et al.*, 1996; Hara *et al.*, 1996). Our findings that p21 increases ROS accumulation, which acts as mediator of the permanent growth arrest phenotype in the absence of continued p21 or p16 expression, provide a possible explanation for these observations. While we show that ROS is necessary for the permanent growth arrest/senescence phenotype induced by p21, our studies do not exclude the importance of the initial growth arrest associated with p21's known cell cycle inhibitory functions. In fact, we have observed that in response to oxidative stress (H_2O_2), wild-type p21 fibroblasts undergo growth arrest but p21^{-/-} fibroblasts fail to arrest and undergo apoptosis at increased H_2O_2 levels (S.Macip, S.Lee and S.Aaronson, unpublished data). This suggests that both p21 growth inhibition and ROS accumulation in response to p21 contribute to the permanent growth arrest phenotype.

It has been established that p21 inhibits cell cycle progression in both G₁ and G₂ through inhibition of

several CDKs (Harper *et al.*, 1993), including CDK2, CDK3, CDK4, CDK6 and CDC2, as well as PCNA, an essential DNA replication factor (Waga *et al.*, 1994). These functions, which reside in different domains, are each sufficient to cause growth arrest (J.Chen *et al.*, 1995; Luo *et al.*, 1995). In contrast, p16 is a member of a family of CDK inhibitors, together with p15, p18 and p19 (Sherr and Roberts, 1995), which specifically inhibit CDK4 and CDK6 and have no effect on PCNA. A p21 mutant, which lacks PCNA-binding ability (Luo *et al.*, 1995; Ando *et al.*, 2001), has been reported to retain the ability to induce growth arrest specifically in G₁, implying that PCNA binding is required for maintenance of the p21 G₂ arrest function (Ando *et al.*, 2001). We showed that this mutant retained the ability to induce both ROS accumulation and permanent growth arrest, demonstrating that p21 binding to PCNA and its G₂ arrest function are not required for these responses.

PIG3, a p53 target gene involved in the ROS pathway, has been proposed to be important for induction of ROS and apoptosis after p53 expression (Polyak *et al.*, 1997). p21 has been implicated in transcription control through interaction with the co-activator p300 (Perkins *et al.*, 1997; Snowden *et al.*, 2000). It has also been proposed that p21 can function as a direct modulator of transcription independently of its CDK inhibition properties (Dotto, 2000), based on the negative role of p21 on differentiation (Di Cunto *et al.*, 1998). We observed that p21 induced up-regulation of PIG3 at both the RNA and protein levels, but that PIG3 levels were not increased in response to p16 expression. An approach to identify additional candidate genes which may influence ROS accumulation in response to p21 currently is being pursued. This approach may help to characterize those genes whose expression alterations are necessary and sufficient for p21-dependent induction of ROS and permanent growth arrest.

Permanent growth arrest/senescence has been proposed to be a tumor suppression mechanism to control the emergence of immortal cells (Sager, 1991; Smith and Pereira-Smith, 1996; Serrano *et al.*, 1997). For instance, p53 (Sugrue *et al.*, 1997) and Rb (Xu *et al.*, 1997) can trigger senescence when reintroduced into immortal tumor cells, which have lost the functions of such genes in the course of tumor evolution. Depending on various factors, induction of p53 after genotoxic stress can result in either senescence or apoptosis, presumably to avoid replication of damaged DNA (Sionov and Haupt, 1999). Our results implicate p21, a known p53 effector, and ROS in the p53 senescent response. The role of ROS in p21-induced senescence underscores the importance of oxidative balance in tumor prevention and aging.

Materials and methods

Cell culture and treatments

EJ cells with a tet-regulated expression system (Fang *et al.*, 1999) were maintained in Dulbecco's modified Eagle's medium (DMEM; Life Technologies) supplemented with 10% fetal bovine serum (FBS), penicillin-streptomycin (50 units/ml), hygromycin (100 μ g/ml) and geneticin (750 μ g/ml), plus 1 μ g/ml tet to repress expression of p21 (EJp21) or p16 (EJp16). Fresh medium with tet was added every 3 days. To induce p21 or p16 expression, cells were washed three times with phosphate-buffered saline (PBS) and seeded in medium in the absence of tet. NAC (Sigma), N-acetyl-L-alanine (Sigma) or GSH (Sigma) were

added to the medium, when required, for the duration of the experiment. 293T cells were maintained in DMEM supplemented with 10% FBS and penicillin–streptomycin (50 U/ml).

Plasmid construction and DNA transfection

EJ-ETH clones that contain the transactivator have been described previously (Sugrue *et al.*, 1997). The p_{tet} puro expression vector was constructed by substitution of the neomycin marker with puromycin. p_{tet}-p16 was constructed by subcloning p16^{Ink4a} cDNA into the *Bam*HI–*Asc*I sites of the p_{tet} puro vector. Transfection with this expression vector into EJ-ETH cells was performed using the standard calcium phosphate method. Transfectants were double selected in the presence of hygromycin (100 µg/ml) and puromycin (2 µg/ml), and individual clones of stable transfectants were selected for further analysis. Transfection with p21 or p21m constructs (generously provided by Y.Xiong) into 293T was performed using the standard calcium phosphate method.

DCF staining

Cells were incubated with 5 µg/ml of DCF (Molecular Probes) for 30 min, then washed with PBS, trypsinized and collected in 1 ml of PBS. Fluorescent stained cells were transferred to polystyrene tubes with cell-strainer caps (Falcon) and subjected to fluorescence-activated cell sorting (FACS; Beckton Dickinson FACScan) using Cell Quest 3.2 (Beckton Dickinson) software for acquisition and analysis.

Cell cycle analysis

Cells were stained with propidium iodide using the CycleTEST Plus DNA reagent kit (Beckton Dickinson), following the instructions provided. Fluorescent stained cells were transferred to polystyrene tubes with cell-strainer caps (Falcon) and subjected to FACS (Beckton Dickinson FACScan) using Cell Quest 3.2 (Beckton Dickinson) software for acquisition and analysis.

Colorimetric assay for glutathione

A colorimetric assay to determine intracellular glutathione concentrations (Oxford Biomedical Research, GT10) was used as described by the manufacturer. Briefly, cells were collected, washed and incubated with the provided reagents, and then the ODs of samples were measured with a spectrophotometer set at 400 nm.

Immunoblot analysis

Cells cultured in the presence or absence of tet were washed twice with ice-cold PBS and lysed in EBC buffer [50 mM Tris pH 8, 120 mM NaCl, 0.5% NP-40, 100 mM sodium fluoride, 2 mM sodium vanadate, 2 mM phenylmethylsulfonyl fluoride (PMSF) and 10 µg/ml aprotinin]. Lysates were cleared by centrifugation at 14 000 r.p.m. for 20 min at 4°C. Protein concentrations were then determined using the BCA protein assay (Pierce). A 40 µg aliquot of total cell protein per sample was subjected to 14% SDS–PAGE and transferred to an Immobilon (Millipore) polyvinylidene difluoride filter. p21 was detected using Ab-1 monoclonal antibody (Oncogene Science) and p16 was detected using C-20 polyclonal antibody (Santa Cruz Biotechnology). Antibodies for PCNA and CDK2 were obtained from Boehringer Mannheim and Upstate, respectively. An enhanced chemiluminescence (ECL) detection system (Amersham) was used.

Immunocytochemistry

Cells were seeded onto glass coverslips and maintained in medium with or without tet for 2–5 days. Coverslips were rinsed with PBS, fixed with 1% paraformaldehyde in PBS for 10 min at room temperature, washed twice with PBS, incubated in pre-cooled 2:1 ethanol/acetic acid for 5 min at –20°C, washed twice with PBS, incubated for 15 min in 3% hydrogen peroxide in PBS and then blocked in normal horse serum for 30 min at room temperature. Cells were stained for p21 using 2.5 µg/ml of Ab-1 monoclonal p21 antibody (Oncogene Science) overnight at 4°C. Following staining, cells were washed twice with PBS and then incubated for 30 min at room temperature in biotinylated horse anti-mouse secondary antibody (Vector Lab). Following two washes with PBS, cells were incubated in streptavidin–horseradish peroxidase (Zymed), washed three more times with PBS and once with 0.5% Triton X-100, and then incubated for 5 min in the dark with diaminobenzidine (DAB; Sigma).

Metabolic labeling, extract preparation and immunoprecipitation analysis

Transfected 293T cells were washed 40–48 h after transfection with 1× PBS and starved for 30 min with DMEM lacking L-methionine/L-cysteine

(Life Technologies) supplemented with 10% dialyzed FBS. Medium was then changed to include 100 µCi/ml Easy Tag Express [³⁵S] Protein Labelling Mix (NEN Life Science) and cells were incubated for 2 h. Following harvesting of the cells by centrifugation, pellets were resuspended in 0.2 ml/plate of buffer A (10 mM Tris–HCl pH 7.4, 10 mM NaCl, 0.5% NP-40, 1 mM PMSF, 2 µg/ml antipain, 2 µg/ml leupeptin) and sonicated (20 s × 6). Buffer B was then added (0.3 ml/plate). The mixture was rotated for 60 min at 4°C and supernatants following centrifugation (100 000 g, 4°C, for 60 min) were saved.

Extracts (100 µl) were then mixed with 10 µg of α-HA (12CA5) and 10 µg of protein A–agarose beads (Upstate Biotechnology) for 1 h at 4°C. The beads were washed three times with 0.5 ml of buffer C (buffers A and B mixed in equal volumes) and then twice with 1× PBS. Bound proteins were released by boiling the beads for 3 min in the presence of 20 µl of 4× concentrated Laemmli loading buffer and separated by 15% SDS–PAGE.

Northern blot analysis

Total RNA was extracted from cells using TriZol (Gibco-BRL). RNA concentrations were measured spectrophotometrically at 260 nm. A 20 µg aliquot of total RNA was electrophoresed at 70 mV for 2–3 h in a 1.6% agarose/formaldehyde gel, transferred to a positively charged nylon membrane and probed with a ³²P-labeled PIG3 probe obtained from the verified IMAGE clone 859359 (supplied by ResGen). After washing once with 2× SSC/0.1% SDS at 50°C, twice with 0.5× SSC/0.1% SDS at 50°C, and twice with 0.1× SSC/0.1% SDS at room temperature, the blots were autoradiographed.

Senescence-associated β-galactosidase (SA-β-gal) staining

Cells were washed in PBS and fixed with 2% formaldehyde/0.2% glutaraldehyde in PBS for 5 min at room temperature. Plates were stained as described previously (Dimri *et al.*, 1995).

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