Cell, Vol. 88, 593–602, March 7, 1997, Copyright ©1997 by Cell Press

Oncogenic *ras* Provokes Premature Cell Senescence Associated with Accumulation of p53 and p16^{INK4a}

Manuel Serrano,*†‡ Athena W. Lin,* Mila E. McCurrach,* David Beach,*† and Scott W. Lowe*
*Cold Spring Harbor Laboratory
†Howard Hughes Medical Institute
Cold Spring Harbor, New York 11724

Summary

Oncogenic *ras* can transform most immortal rodent cells to a tumorigenic state. However, transformation of primary cells by *ras* requires either a cooperating oncogene or the inactivation of tumor suppressors such as p53 or p16. Here we show that expression of oncogenic *ras* in primary human or rodent cells results in a permanent G1 arrest. The arrest induced by *ras* is accompanied by accumulation of p53 and p16, and is phenotypically indistinguishable from cellular senescence. Inactivation of either p53 or p16 prevents *ras*-induced arrest in rodent cells, and E1A achieves a similar effect in human cells. These observations suggest that the onset of cellular senescence does not simply reflect the accumulation of cell divisions, but can be prematurely activated in response to an oncogenic stimulus. Negation of *ras*-induced senescence may be relevant during multistep tumorigenesis.

Introduction

The *ras* family of proto-oncogenes encodes small GTP-binding proteins that transduce mitogenic signals from tyrosine-kinase receptors (reviewed by Barbacid, 1987; Cahill et al., 1996). Amplification of *ras* proto-oncogenes and mutations that constitutively activate Ras proteins are frequent in human cancers (reviewed by Bos, 1988). Oncogenic *ras* efficiently transforms most immortal rodent cell lines but fails to transform primary cells (Newbold and Overell, 1983). *ras* cooperates, however, with certain other oncogenic alterations to transform primary rodent cells (Land et al., 1983; Ruley, 1983), including overexpression of c-Myc, dominant negative p53, D-type cyclins, and Cdc25A and -B, and loss of *p53*, *p16*, or *IRF-1* (reviewed by Weinberg, 1989; Ruley, 1990; see also Hinds et al., 1994; Lovec et al., 1994; Tanaka et al., 1994; Galaktionov et al., 1995; Serrano et al., 1996). Several viral oncoproteins also cooperate with *ras*, including SV40 T-antigen, adenovirus E1A, human papillomavirus E7, and HTLV-1 Tax (Weinberg, 1989; Ruley, 1990). When expressed alone, most of these cooperating alterations facilitate the establishment of primary cells into immortal cell lines (Ruley, 1990). Oncogenic transformation of primary cells by coexpression of *ras* and "immortalizing" mutations constitutes a model of multistep tumorigenesis that has been reproduced in animal systems (reviewed by Hunter, 1991; Vogelstein and Kinzler, 1993; see also Kemp et al., 1993).

Together with *ras* mutations, inactivation of the tumor suppressors *p53* and *p16* are the most prevalent mutations in human tumors (reviewed by Hirama and Koeffler, 1995; Hollstein et al., 1996). Mice with *p53* gene deletions develop normally but are highly prone to tumor development (Donehower et al., 1992; Jacks et al., 1994). Indeed, p53 is not required for normal cell growth, but acts to prevent proliferation under circumstances of cellular stress. Hence, the normally low levels of p53 increase following DNA damage, certain oncogenic insults, hypoxia, and a variety of other cellular stresses (reviewed by Ko and Prives, 1996). Activation of p53 prevents cell proliferation by inducing either cell-cycle arrest or apoptosis. A variety of factors determine the probability of a particular outcome, including cell type, Rb activity, ectopic expression of certain oncogenes, and levels of proteins such as Bax, Bcl-2, and others (Ko and Prives, 1996; see also Lowe et al., 1993; McCurrach et al., 1997). p53 is a sequence-specific DNA-binding protein that activates transcription of genes involved in cell-cycle arrest and in apoptosis. The expression of the cell-cycle inhibitor p21 is transcriptionally activated by p53 and contributes to p53-dependent arrest (Ko and Prives, 1996).

p16^{INK4a} encodes a protein that associates with CDK4 and inhibits its activity (Serrano et al., 1993; Xiong et al., 1993), and is mutated in a wide variety of cancers (reviewed by Hirama and Koeffler, 1995). Like p53, p16 has no obvious role in normal development or intrinsic cell-cycle control but is a potent tumor suppressor. Hence, *p16*-deficient mice are viable but highly prone to spontaneous and carcinogen-induced tumors (Serrano et al., 1996). The primary function of the CDK4/cyclin D kinases is to phosphorylate the retinoblastoma tumor suppressor protein, Rb, allowing progression of the cell-cycle toward S phase (reviewed by Sherr and Roberts, 1995; Weinberg, 1995). Thus, expression of p16 inhibits CDK4/D kinases and leads to G1 arrest, but is inconsequential in the absence of functional Rb. In contrast to p53, little is known about the regulation of p16 expression or activity, or about circumstances in which p16 might act physiologically to promote cell-cycle arrest.

Primary murine fibroblasts lacking either p53 or p16 are transformed by oncogenic *ras* alone (Tanaka et al., 1994; Serrano et al., 1996) and are easily established into immortal cell lines (Harvey et al., 1993; Metz et al., 1995; Serrano et al., 1996). In human cells, inactivation of p53 and/or p16 also facilitates the immortalization process (Reznikoff et al., 1996; Rogan et al., 1996). Together, these observations suggest a role for p53 and p16 in cellular senescence and, consistent with this, p53, p21, and p16 levels and/or activity increase in senescent cells (Alcorta et al., 1996; Hara et al., 1996; Reznikoff et al., 1996). In this study, we demonstrate that prolonged expression of oncogenic *ras* induces a cell-cycle arrest involving p53 and p16 that is indistinguishable from cellular senescence. We suggest that premature senescence is a mechanism of tumor suppression that can

‡Present Address: Department of Immunology and Oncology, Centro Nacional de Biotecnología (CSIC), Campus de Cantoblanco, Madrid, E-28049, Spain.

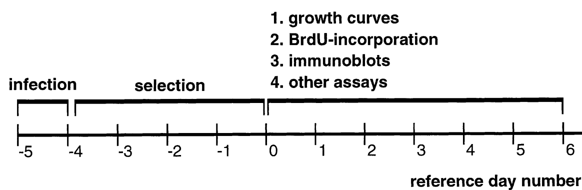

Figure 1. Experimental Design and Reference Time Frame

explain several aspects of *ras* transformation and may be an important safeguard against neoplasia.

Results

Oncogenic *ras* Provokes Cell Arrest

We chose three well-characterized systems to examine the effects of oncogenic *ras* in normal cells: primary (presenescent) human diploid fibroblasts (IMR90 and WI38), primary mouse embryo fibroblasts (MEFs), and the rat REF52 cell line. REF52 cells are immortal fibroblasts but are similar to primary cells in that they display a high degree of growth control and require coexpression of a cooperating oncogene for transformation by *ras* (Franza et al., 1986). An activated *ras* allele (H-*ras*V12) was introduced into these cells using recombinant replication-deficient retroviruses as described in Experimental Procedures. The percentage of retrovirally transduced cells ranged between 60% and 100%, as estimated in parallel infections using viruses expressing the *lacZ* gene product. The retroviral vectors coexpressed a puromycin phosphotransferase gene that allowed selection for pure populations of transduced cells within four days. The fifth day postinfection is designated day 0 in all the following experiments (Figure 1). Using this protocol, we observed an approximate 5- to 10-fold increase in total Ras protein, compared to endogenous Ras levels (data not shown).

Expression of H-*ras*V12 produced morphological changes in human IMR90 and WI38 cells. Initially, cells displayed a refractile cytoplasm with thin and long projections (data not shown). By day 2, a significant proportion of H-*ras*V12-transduced cells became flat and enlarged; by day 6, virtually all cells had acquired the enlarged morphology and apparently were growth arrested (Figure 2A; data not shown for WI38). Similar changes in cell morphology were observed in primary MEFs and REF52 cells (Figure 2A; data not shown for WI38). No changes in morphology were detected in cell populations transduced with empty viral vector (Figure 2A).

The proliferative properties of such cell populations were monitored by growth curves and by BrdU-labeling, followed by flow cytometry. Cells transduced with H-*ras*V12 or with empty vector were plated at low density and cell numbers were counted (Figure 2B; data not shown for WI38 cells). Control populations grew steadily and reached confluence by day 6 (Figures 2A and 2B). By contrast, H-*ras*V12-transduced cells stopped accumulating well before reaching confluence (Figures 2A and 2B). BrdU incorporation combined with DNA-content analysis revealed that H-*ras*V12-transduced populations retained a small percentage of BrdU-positive cells at day 1, but virtually none by day 3 (Figure 2C). H-*ras*V12-transduced cells accumulated preferentially in G1, with a cell-cycle profile indistinguishable from serum-starved control cells (Figure 2C; data not shown). The proportion of BrdU-positive cells in control populations was high, and declined only when cells reached confluence between days 3 and 5 (Figure 2C). Similarly, MEFs and REF52 cell populations transduced with H-*ras*V12 displayed reduced BrdU incorporation compared to vector-transduced controls (see below). Hence, the inability of the H-*ras*V12-transduced cells to proliferate is primarily due to cell-cycle arrest rather than cell death. Cells arrested by oncogenic *ras* remained attached to the plates and retained the same flat and enlarged morphology for at least one month (data not shown). Thus, *ras* induces cell-cycle arrest prior to the onset of S phase, and this program is conserved between human and rodent fibroblasts.

Cell-Cycle Proteins

To characterize the nature of the cell-cycle arrest caused by H-*ras*V12, we examined the expression of several cell-cycle regulatory proteins by immunoblotting (Figure 3A). Cell lysates were prepared between days 2 and 4. In all three experimental systems, oncogenic *ras* resulted in elevated p53 and p21. Furthermore, oncogenic *ras* also increased p16 levels. On average, p53, p21, and p16 increases ranged between 10- and 20-fold, compared to control cells and relative to the total cellular protein content. Declines in the levels of hyperphosphorylated Rb, cyclin A, and CDK2 kinase activity were also observed in H-*ras*V12-transduced cells (Figures 3A and 3B). Moderate increases in the levels of cyclin D1 were also observed in the presence of H-*ras*V12 (data not shown), in agreement with previous reports using immortal cell lines (Liu et al., 1995).

Decreases in the abundance of hyperphosphorylated Rb, cyclin A, and CDK2 activity are characteristic features of G0/G1 arrest; for example, during quiescence as a result of serum-starvation or confluence (see Dulic et al., 1993). By contrast, neither p53 nor p16 accumulate during quiescence (Afshari et al., 1993; Alcorta et al., 1996). Consequently, the G1 arrest induced by oncogenic *ras* is different from quiescence and is specifically characterized by the simultaneous induction of the p53 and p16 tumor suppressor proteins.

Cellular Senescence

The features of H-*ras*V12-arrested cells described above are remarkably similar to cells that have surpassed their proliferative capacity and become senescent (Hayflick and Moorhead, 1961). For example, senescent cells adopt a flat enlarged morphology and cease proliferation at subconfluent densities despite the presence of serum (reviewed by Stein and Dulic, 1995; Votja and Barrett, 1995). Also, senescent cells arrest with a G1 DNA content, and express increased levels and/or activity of p53, p21, and p16 (Noda et al., 1994; Atadja et al., 1995; Alcorta et al., 1996; Hara et al., 1996; Reznikoff et al., 1996). To exlore further the potential relationship between *ras*-induced arrest and senescence, we examined three additional senescence-associated markers: lack of serum-dependent induction of

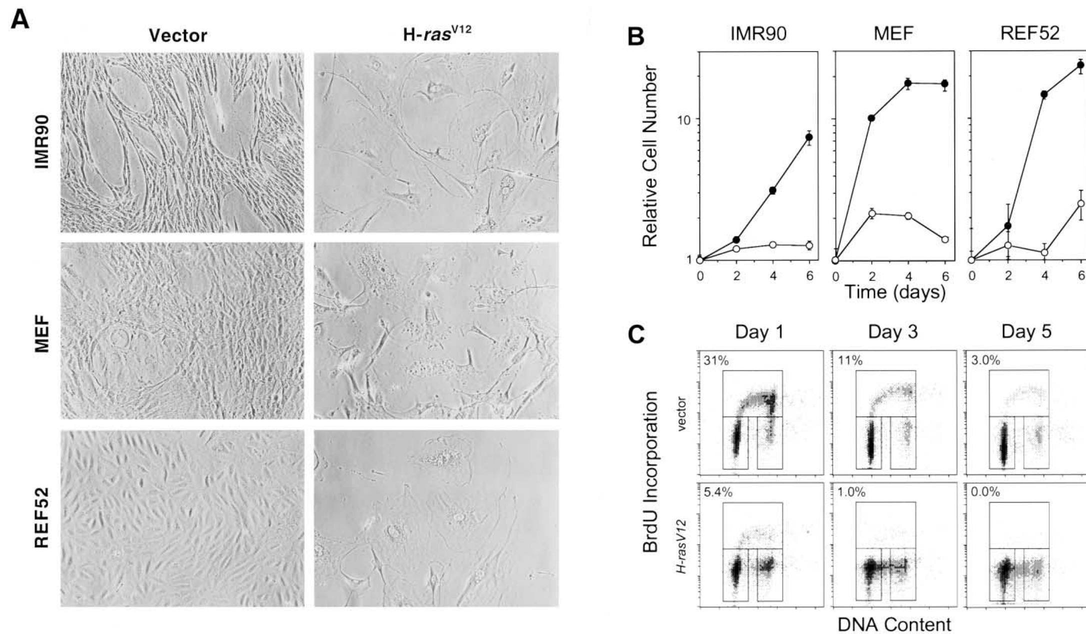

Figure 2. Effect of H-*ras*V12 on Primary Fibroblasts

The indicated cell-types were transduced with retroviruses expressing H-*ras*V12 or with empty-vector control (pBabe-Puro).

(A) Cell morphology at day 6. Photographs are at the same magnification.

(B) Representative growth curves corresponding to the indicated cell cultures transduced with empty vector (closed circles) or with H-*ras*V12-expressing (open circles) retroviruses. The time frame corresponds to the scheme in Figure 1. Each curve was performed at least twice, and each time point was determined in triplicate.

(C) Cell-cycle distribution of H-*ras*V12-expressing and control populations as measured by BrdU incorporation and DNA-content flow cytometry analysis. Cells were stained with FITC-anti-BrdU to detect BrdU incorporation (vertical axis) and propidium iodide to detect total DNA (horizontal axis). The upper box identifies cells incorporating BrdU (~S phase), the lower-left box identifies G0/G1 cells, and the lower-right box displays G2/M cells. The percentage of cells incorporating BrdU is indicated in the upper left of each graph.

c-*fos* (Seshadri and Campisi, 1990), overexpression of the plasminogen activator inhibitor type-1 (*PAI-1*) gene (Goldstein et al., 1994; Mu and Higgins, 1995), and detection of a senescence-associated β-galactosidase (SA-β-gal) optimally active at a pH of 6.0 (Dimri et al., 1995; see also Reznikoff et al., 1996).

To test induction of c-*fos* mRNA, IMR90 cells transduced with H-*ras*V12 or with empty vector were serum starved from days 0 to 4, and then restimulated with serum. As expected, vector-transduced cells transiently induced c-*fos* 30 min after restimulation (Figure 4A). No c-*fos* message was detected, however, in H-*ras*V12-transduced cells (Figure 4A). PAI-1 regulates cell-extracellular matrix interactions and is translated from two mRNAs. During senescence, both *PAI-1* transcripts become elevated, but the increase in expression of the small transcript is more pronounced (Mu and Higgins, 1995). Likewise, H-*ras*V12-transduced IMR90 cells displayed a significant accumulation of *PAI-1* mRNA, compared with vector-transduced cells (2.5-fold large transcript; 7.5-fold small transcript) (Figure 4B).

The SA-β-gal activity was assessed at several times postinfection (Figure 4C). The percentage of SA-β-gal-positive cells in H-*ras*V12-expressing populations was initially low, but increased at day 3 and reached 60% by day 6 (Figures 4C and 4D). In contrast, about 3% of the IMR90 cells in control cultures were SA-β-gal positive, a value consistent with that previously described for young IMR90 cells (Dimri et al., 1995). Cell-cycle arrest produced by serum depletion or confluence produced no increase in the percentage of SA-β-gal-positive cells in IMR90 control cells (Figure 4C and data not shown). Thus, the cell-cycle arrest induced by oncogenic *ras* was identical to replicative senescence by every criterion tested.

E1A Bypass of Arrest

In rodent fibroblasts, the adenovirus *E1A* oncogene cooperates with H-*ras*V12 to transform primary cells oncogenically (Ruley, 1983). To determine whether E1A could circumvent *ras*-induced cell-cycle arrest, E1A was transduced into MEF, REF52, and IMR90 cell populations using a vector coexpressing *E1A 12S* and hygromycin phosphotransferase. After selection in hygromycin, *E1A*-expressing cells were infected with an H-*ras*V12-expressing or control virus, according to the scheme in Figure 1. In each cell type, *E1A*-expressing fibroblasts transduced with H-*ras*V12 incorporated BrdU with efficiencies comparable to those of cells transduced with empty vector, and proliferated rapidly (Figures 5 and 6). These results indicate that *E1A* efficiently negates the cell-cycle arrest induced by oncogenic *ras*, and are consistent with the observation that *E1A* cooperates with *ras* in transformation assays. Importantly, the levels of H-Ras proteins following introduction of H-*ras*V12 were similar between parental and *E1A*-expressing cells (data not shown), demonstrating that overexpression of H-*ras*V12 is not necessarily detrimental to cell proliferation.

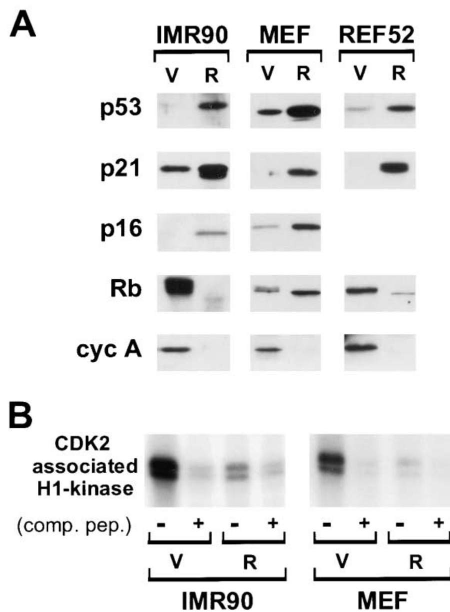

Figure 3. Effect of H-*ras*V12 on Cell-Cycle Regulatory Proteins

(A) Immunoblots of cellular lysates corresponding to cells transduced with empty vector (V) or with H-*ras*V12-expressing (R) retroviruses. Our anti-p16 antibodies did not crossreact with rat p16, so we could not examine p16 expression in REF52 cells.

(B) CDK2 kinase activity against histone H1 obtained after immunoprecipitation of the indicated cellular lysates, (V) or (R) as in (A), with anti-CDK2 in the absence (−) or presence (+) of competing antigenic peptide.

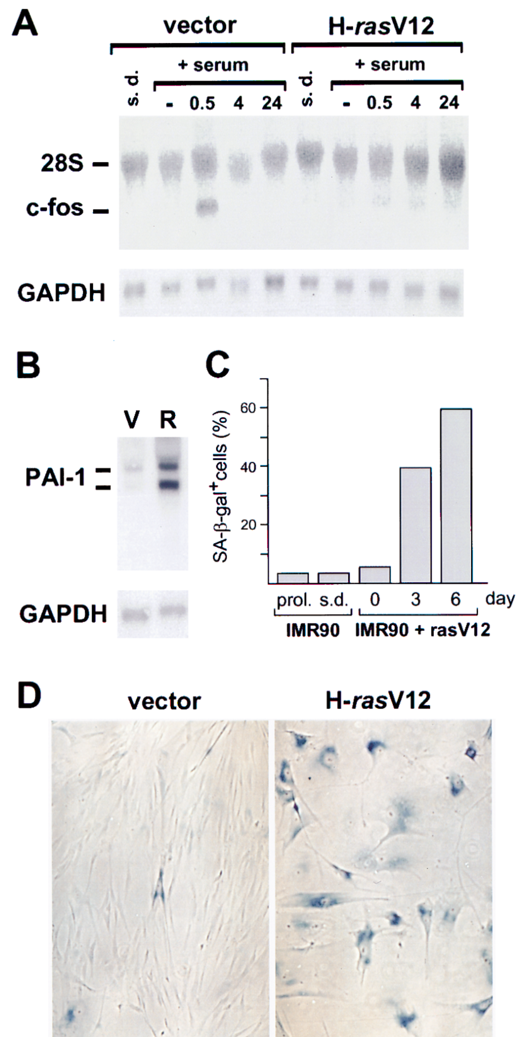

Figure 4. Senescent Features of the H-*ras*V12-Transduced Cells

(A) Expression of c-*fos* in response to serum restimulation in IMR90 cells transduced with empty vector or with H-*ras*V12-expressing retroviruses. At day 0 (see Figure 1), cultures were serum-deprived (s.d.) in 0.5% serum for 96 hr and then restimulated by addition of 10% serum. At the indicated times after restimulation, total RNA was prepared and probed with c-*fos* and GAPDH as a loading control. The c-*fos* probe crossreacted with 28S ribosomal RNA.

(B) Expression of plasminogen activator inhibitor type-1 (*PAI-1*) in IMR90 cells transduced with empty vector (V) or with H-*ras*V12-expressing (R) retroviruses at day 6. The two transcripts of *PAI-1* are indicated (see Mu and Higgins, 1995).

(C) β-gal (pH 6.0) activity in proliferating (prol.) or serum-deprived (s.d.) IMR90 cells, and in cells transduced with H-*ras*V12-expressing retrovirus at the indicated days.

(D) Photographs of the indicated IMR90 cells stained for β-gal (pH 6.0) activity at day 6. Photographs are at the same magnification.

Both MEF and IMR90 cells expressing *E1A* alone displayed a normal rate of BrdU incorporation, but cell numbers accumulated slowly (compare Figures 5A and 5C with Figures 6A and 6C, respectively). This discrepancy presumably reflects a high rate of apoptosis occurring in the *E1A*-expressing cells (Lowe et al., 1994). Notably, primary MEFs and REF52 cells expressing *E1A* were morphologically altered and appeared oncogenically transformed following introduction of H-*ras*V12 (data not shown). *E1A* also altered the morphology of IMR90 cells. In contrast to rodent cells, however, IMR90 cells expressing *E1A* and transduced with H-*ras*V12 remained growing as monolayers and displayed no indication of neoplastic transformation (see Figure 7B).

Disruption of p53 or p16/Rb Pathways

To examine the independent contribution of p53 and p16 to *ras*-induced cell-cycle arrest, the response of wild-type MEFs to oncogenic *ras* was compared to otherwise identical MEFs derived from *p53*- or *p16*-deficient mice (Jacks et al., 1994; Serrano et al., 1996). In contrast to wild-type MEFs, $p53^{-/-}$ or $p16^{-/-}$ MEFs continued to incorporate BrdU and proliferate following introduction of H-*ras*V12 (Figures 5A and 6A). In agreement with previous reports (Tanaka et al., 1994; Serrano et al., 1996), both $p53^{-/-}$ and $p16^{-/-}$ MEFs expressing H-*ras*V12 displayed features of oncogenic transformation (e.g., refractile morphology, loss of contact inhibition), which were apparent almost immediately after H-*ras*V12 was transduced (data not shown). These results indicate that p53 and p16 are essential for *ras*-induced arrest in MEFs, and that inactivation of either p53 or p16 alone is sufficient to circumvent arrest.

In REF52 and IMR90 fibroblasts, a different approach was needed to disrupt p53 or the p16/Rb pathway. To inactivate p53 specifically, we transduced $p53^{175H}$, a dominant-negative p53 mutant, in the same manner as

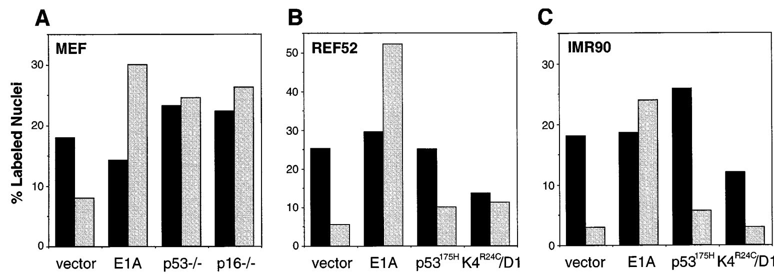

Figure 5. Genetic Analysis of H-*ras*V12-Induced Arrest: BrdU Incorporation at Day 2

The indicated cell types were transduced with empty vector (solid bars) or with H-*ras*V12-expressing (shaded bars) retroviruses. BrdU incorporation was detected by immunofluorescence. The average of two experiments is shown, and at least 200 cells were counted per experiment.

(A) Mouse embryo fibroblasts (MEF) of wild-type genotype containing empty vector or expressing E1A, MEF-$p53^{-/-}$, or MEF-$p16^{-/-}$, as indicated.

(B) REF52 cells containing empty vector or expressing the indicated proteins.

(C) IMR90 cells containing empty vector or expressing the indicated proteins.

was described for E1A (see Experimental Procedures). Transduction of dominant-negative p53 inactivated endogenous p53, as judged by its ability to abolish p21 expression (data not shown). To disrupt the p16/Rb pathway, we sequentially transduced cyclin D1 and $CDK4^{R24C}$ using different selectable markers (see Experimental Procedures). $CDK4^{R24C}$ is a mutant CDK4 protein insensitive to p16 (Wölfel et al., 1995), and cooperates with cyclin D1 to prevent cell-cycle arrest induced by p16 overexpression (Bartkova et al., 1996; J. Koh and E. Harlow, personal communication). The cyclin D1/$CDK4^{R24C}$ complexes are expected to inactivate the p16/Rb pathway by phosphorylating Rb in a p16-insensitive manner, and possibly by sequestering other cell-cycle inhibitors such as p21 and p27.

Disruption of p53 activity compromised *ras*-induced arrest in REF52 cells but not in IMR90 cells. Specifically, REF52/$p53^{175H}$ populations transduced with H-*ras*V12 showed a partial decrease in BrdU incorporation at day 2, but eventually outgrew the vector-transduced REF52/$p53^{175H}$ (Figures 5B and 6B). Concordant with previously published results (Hicks et al., 1991), REF52 cells coexpressing mutant *p53* and *ras* appeared morphologically transformed (data not shown). In contrast, IMR90/$p53^{175H}$ cells transduced with H-*ras*V12 underwent cell-cycle arrest and displayed the large flat morphology characteristic of the parental cells expressing H-*ras*V12 alone (Figures 5C and 6C, and data not shown). Thus, whereas p53 is essential for *ras*-induced arrest in rodent fibroblasts, it appears dispensable in human fibroblasts.

Similarly, disruption of the p16/Rb pathway by cyclin D1/$CDK4^{R24C}$ allowed escape from *ras*-induced arrest in REF52 but not in IMR90 fibroblasts. Hence, in agreement with the behavior of $p16^{-/-}$ MEFs, REF52 cells coexpressing cyclin D1 and p16-insensitive $CDK4^{R24C}$ continued incorporating BrdU and proliferating upon introduction of H-*ras*V12 (Figures 5B and 6B). Moreover, these cells became small and refractile, and showed loss of contact inhibiton, which are both characteristics of neoplastic transformation (data not shown). In contrast, transfer of H-*ras*V12 into IMR90 populations coexpressing cyclin D1 and $CDK4^{R24C}$ elicited the same changes in cell morphology and growth as in control IMR90 cells. Thus, cells became large and flat, and displayed a significant decrease in BrdU incorporation and growth rate (Figures 5C and 6C). In a parallel situation to that described for p53, disruption of the p16/Rb pathway is sufficient to prevent *ras*-induced arrest in rodent but not in human fibroblasts.

Absence of Senescence-Associated Markers in Cells Coexpressing *E1A* and Oncogenic *ras*

To test further the significance of the senescence markers induced by H-*ras*V12 in IMR90 cells (see above), we examined the behavior of these markers in IMR90 cells expressing *E1A* and transduced with H-*ras*V12. H-*ras*V12 failed to induce *PAI-1* expression or SA-β-gal activity in IMR90 cells expressing *E1A* (Figures 7A and 7B). The response of c-*fos* to serum stimulation could not be assessed because *E1A*-expressing cells undergo apoptosis following serum depletion (Lowe and Ruley, 1993; Lowe et al., 1994). These results indicate that overexpression of *PAI-1* and the presence of SA-β-gal activity are not direct consequences of oncogenic *ras*, but rather are associated with the arrested state induced by *ras*. In this regard, our observations further strengthen the association of these markers with cellular senescence.

Discussion

The effect of *ras* on proliferation and tumorigenesis has been well documented in the context of immortal cell lines (Barbacid, 1987; Cahill et al., 1996). However, the analysis of the effects of prolonged *ras* expression in primary cells has remained largely unexplored, mainly due to the limited lifespan of primary cells and to the low efficiencies of conventional gene transfer methods. Retrovirus-mediated gene-transfer allows the introduction of exogenous genes with high efficiencies, such that the effects of a transduced gene can be assessed in whole cell populations without significant expansion of the culture. We took advantage of these methods to study the effects of oncogenic *ras* in primary cells.

Cell-Cycle Arrest Induced by Oncogenic *ras*

We show that *ras* provokes a permanent cell-cycle arrest in primary fibroblasts of rodent and human origin. The arrest occurs at G1 and in association with a significant increase in the abundance of p53, p21, and p16. In this regard, oncogenic activation of *ras* constitutes a novel circumstance in which p53 is activated, and provides a

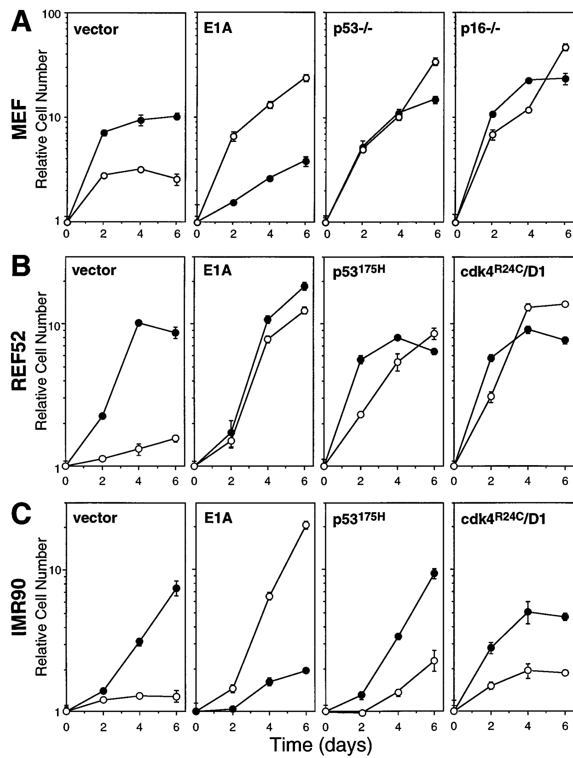

Figure 6. Genetic Analysis of H-*ras*V12-Induced Arrest: Growth Curves

The indicated cell types were transduced with empty vector (closed circles) or with H-*ras*V12-expressing (open circles) retroviruses. A representative experiment is shown. Each experiment was performed at least twice, and each time point was determined in triplicate.

(A) Mouse embryo fibroblasts (MEF) of wild-type genotype containing empty vector or expressing E1A, MEF-*p53*$^{-/-}$, or MEF-*p16*$^{-/-}$, as indicated.

(B) REF52 cells containing empty vector or expressing the indicated proteins.

(C) IMR90 cells containing empty vector or expressing the indicated proteins.

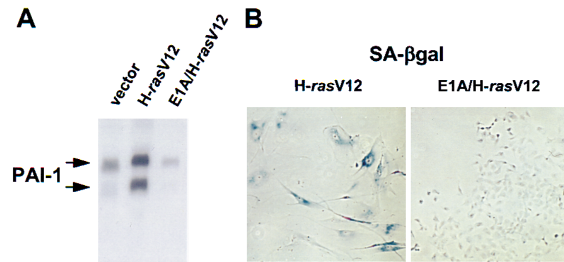

Figure 7. Absence of Senescence Markers in IMR90 Cells Expressing *E1A* and H-*ras*V12

(A) Expression of plasminogen activator inhibitor type-1 (PAI-1) at day 6 in IMR90 cells containing empty vector or expressing H-*ras*V12 alone, or in combination with *E1A*, as indicated. The two transcripts of *PAI-1* are indicated.

(B) Photographs of the indicated IMR90 cells stained for β-gal (pH 6.0) activity at day 6. Photographs are at the same magnification.

description of a stimulus that can induce p16 in association with cell-cycle arrest. It has been previously reported that cells lacking functional Rb overexpress p16 (Serrano et al., 1993; Hara et al., 1996). However, the absence of functional Rb renders the cells insensitive to p16 and cells proliferate in the face of high levels of p16 protein.

In rodent fibroblasts, disruption of either *p53* or *p16* alone is sufficient to prevent *ras*-induced arrest. p53 and p16 are not thought to promote cell-cycle arrest through the same pathway; consistent with this, p16 levels increase in response to *ras* in *p53*$^{-/-}$ MEFs, and p53 levels increase in *p16*$^{-/-}$ MEFs (data not shown). Thus, *ras*-induced accumulation is achieved by the two proteins independently of one another and, at the levels achieved, neither alone is sufficient to promote arrest. E1A is a viral oncoprotein known to inactivate Rb and to override p53-mediated arrest (Lowe et al., 1993). In normal human fibroblasts, by contrast, independent disruption of either p53 or the p16/Rb pathway alone is not sufficient to prevent *ras*-induced arrest, but the E1A oncoprotein allows escape from *ras*-induced arrest. The differential sensitivity between human and rodent cells to individual perturbation of p53 or the p16/Rb pathway may underlie the well-known difficulty in establishing and transforming human cell lines in culture.

Several observations indicate that *ras*-induced arrest is not an immediate outcome of Ras signaling, but rather a cellular response to aberrant Ras activity. The immediate effect of Ras in primary cells is mitogenic, as indicated by microinjection studies in primary human and rodent fibroblasts (Lumpkin et al., 1986; Sullivan et al., 1986; Serrano et al., 1995). Also, the cell-cycle arrest induced by *ras* in our studies is not immediate but occurs after a lag period, typically becoming complete by day 2 of the experimental scheme shown in Figure 1 (day 7 after the intitial introduction of H-*ras*V12). This delayed response is consistent with the existence of a homeostatic mechanism that prevents proliferation after prolonged Ras activation and that is mediated, at least in part, by the induction of p53 and p16.

Antiproliferative responses to oncogenic *ras* are not unprecedented. Oncogenic *ras* has been reported to result in proliferation arrest in REF52 fibroblasts (Franza et al., 1986; Hirakawa and Ruley, 1988) and in primary rat Schwann cells (Ridley et al., 1988). Oncogenic *ras* also induces proliferative arrest and differentiation in two rodent cell lines, rat pheochromocytoma PC12 cells and mouse NIH-3T3-L1 fibroblasts (Bar-Sagi and Feramisco, 1985; Benito et al., 1991; Marshall, 1995). However, it is important to point out that microinjection of Ras into Schwann and PC12 cells immediately results in proliferative arrest or differentiation, respectively (Bar-Sagi and Feramisco, 1985; Ridley et al., 1988). This is in contrast to primary fibroblasts, where microinjection of Ras initially results in proliferation (Lumpkin et al., 1986; Sullivan et al., 1986) and probably reflects cell–type specific differences.

Senescence Induced by Oncogenic *ras*

The cell-cycle arrest induced by oncogenic *ras* is phenotypically similar, and perhaps identical, to cellular senescence. Senescence was originally defined by the observation that primary fibroblasts have a limited proliferative capacity that is genetically determined (Hayflick

and Moorhead, 1961; Röhme, 1981; Votja and Barrett, 1995). At the end of their lifespan, cells undergo changes in morphology, physiology, and gene expression, and permanently cease proliferation (Stein and Dulic, 1995). In this state, cells remain metabolically active for long periods of time.

Under normal culture conditions, induction of senescence depends solely on the accumulated number of cell doublings. However, a phenotype suggestive of premature senescence can be induced following exposure of cell cultures to DNA-demethylating drugs (Holliday, 1986; Fairweather et al., 1987), ceramide (Venable et al., 1995), or inhibitors of histone deacetylation (Ogryzko et al., 1996). In addition, the existence of multiple potential pathways that can promote senescence is supported by cell fusion studies (Votja and Barrett, 1995). Based on our observations, we envision that senescence occurs not merely as a passive consequence of a cell-division counting mechanism, but rather as a dynamic response to an aggressive mitogenic stimulus. In this regard, it is possible that the normal Hayflick limit reflects the accumulated mitogenic load that occurs during the lifetime of a cell culture.

Oncogene Cooperation and Multistep Carcinogenesis

Disruption of p53 or the p16/Rb pathway in rodent cells is extremely efficient at abrogating *ras*-induced arrest. In fact, oncogenic *ras* alone transforms primary mouse fibroblasts deficient in either *p53* or *p16* (Tanaka et al., 1994; Serrano et al., 1996). Hence, escape from *ras*-induced arrest may be sufficient for transformation by oncogenic *ras*. Other alterations known to cooperate with *ras* also disrupt p53 or the p16/Rb pathway. For example, T-antigen inactivates p53 and Rb; cyclin D1 inactivates Rb by promoting its phosphorylation; the viral oncoprotein Tax inactivates p16 (Suzuki et al., 1996); the Cdc25A and -B phosphatases can activate CDK2, leading to Rb inactivation; and, finally, Myc activates the expression of Cdc25A and -B (Galaktionov et al., 1996). We propose that the cell-cycle activation associated with disruption of p53 or the p16/Rb pathway is necessary for transformation by oncogenic *ras*.

The induction of a premature senescence program by oncogenic *ras* provides an explanation for the cooperation between *ras* and the immortalizing mutations that transform primary rodent cells (see Introduction). Mutations that facilitate immortalization may disable not only the senescence program associated with the accumulation of cell doublings but also, and perhaps more importantly, they may impair *ras*-induced senescence. Similarly, most spontaneously immortalized rodent cells can be transformed by *ras* alone, suggesting that *ras*-induced senescence is impaired. Indeed, mutations in *p53* and *p16* often occur spontaneously during the establishment of immortal cell lines (Harvey and Levine, 1991; Reznikoff et al., 1996; Rogan et al., 1996). The H-*ras*V12 allele used in this study was originally isolated from tumor-derived DNA by its ability to transform NIH-3T3 cells, an immortal rodent cell line (Barbacid, 1987). Interestingly, it is now known that NIH-3T3 cells have deleted the *p16* gene (Quelle et al., 1995; Linardopoulos et al., 1995).

Cell-cycle arrest in response to oncogenic *ras* may provide selective pressure to mutate *p53* and *p16* during carcinogenesis. Consistent with this view, mutation and/or amplification of K-*ras* and loss of *p16* and *p53* are extremely common in human pancreatic cancers (Bos, 1988; Caldas et al., 1994). In colorectal cancer, mutation and/or amplification of K-*ras* typically precedes *p53* mutation (reviewed by Kinzler and Vogelstein, 1996). In animal models, *p53* deficiency accelerates tumor progression in *ras*-transgenic mice (Kemp et al., 1993), and mice lacking p16 rapidly develop malignant tumors by treatment with DMBA (Serrano et al., 1996), a carcinogen that efficiently mutates H-*ras* (reviewed by Barbacid, 1987).

It is possible that the effects of oncogenic *ras* expression that we observed may not precisely reflect cellular responses to *ras* mutation in vivo. Of note, we introduced *ras* into whole cell populations and, consequently, Ras levels among individual cells may vary. Yet, despite this intrinsic variability, the individual cell responses are remarkably uniform: the vast majority of cells arrest upon transduction of *ras*, and the arrest program is conserved between fibroblasts from three species. A similar variability in *ras* expression is also presumed in tumors, particularly in those tumors where *ras* proto-oncogenes are amplified (Bos, 1988). We speculate that the intensity of the *ras* oncogenic stimulus may affect the lag period that precedes the induction of the cellular antiproliferative response described here.

Concluding Remark

We demonstrate that oncogenic *ras* provokes premature senescence in primary rodent and human cells in association with the induction of p53 and p16. We propose that premature induction of senescence in response to abnormal mitogenic signaling is a mechanism of tumor suppression. Inactivation of this antiproliferative response allows proliferation to continue unabated in the presence of oncogenic stimuli. These observations could explain the apparent pressure to mutate genes involved in the establishment of oncogene-induced senescence, such as *p53* and *p16*, during tumor progression.

Experimental Procedures

Retroviral Vectors

The following plasmids were used for generating retroviruses: pBabe (Morgenstern and Land, 1990); pLPC (coexpresses the gene of interest from an internal CMV promoter with a puromycin resistance gene driven by the LTR); and pWZL (coexpresses the gene of interest with a selectable marker translated from an internal ribosomal entry site) (a gift of J. Morgenstern). Oncogenic *ras* was transduced using a pBabe-Puro-based vector expressing a human H-*ras*V12 cDNA (pBabe-ras), although transduction of H-*ras*V12 using pLPC, pWZL-Hygro, or pWZL-CD8 (pWZL8ras coexpressing a CD8 cell-surface marker) gave similar results (data not shown). pWZL-Hygro derivatives were used to generate retroviruses expressing the following proteins: an adenovirus-5 12S cDNA (pWZL12S); a human $p53^{175H}$ mutant (pWZLp53.175); and the p16-insensitive $CDK4^{R24C}$ mutant (pWZLR24C). A pWZL-Neo derivative was used to express the ecotropic retroviral receptor (pWZL-Neo-EcoR), and a pLPC derivative was used to express cyclin D1 (pLPCD1).

Cell Culture and Preparation of MEFs

Human primary fibroblasts (IMR90, WI38) and an amphotrophic retrovirus packaging line (BING) were obtained from ATCC. REF52

cells were obtained from Cold Spring Harbor Laboratory. The ecotropic virus packaging line Phoenix was obtained from G. Nolan. MEFs were prepared from day 13.5 embryos derived from crosses between either *p53*$^{+/-}$ mice (Jacks et al., 1994) or *p16*$^{+/-}$ mice (Serrano et al., 1996). The head and the red organs were removed, then the torso was minced and dispersed in 0.1% trypsin (45 min at 37°C). Cells were grown for two population doublings and then frozen. The wild-type MEFs used for comparison were derived from the same crosses, and their behavior was identical regardless of their provenance (*p53*$^{+/-}$ cross or *p16*$^{+/-}$ cross). Human fibroblasts were used between 20 and 30 population doubling levels (PDLs); MEFs between 3 and 5 PDLs. All cultures were maintained in DMEM (GIBCO) plus 10% fetal bovine serum (FBS; Sigma). Primary human and mouse fibroblasts were subcultured 1:4 upon reaching confluence; each passage was considered two PDLs.

Retroviral-Mediated Gene Transfer

Phoenix cells (5×10^6) were plated in a 10 cm dish, incubated for 24 hr, and then transfected by calcium-phosphate precipitation with 20 μg of a retroviral plasmid (15 hr at 37°C). After 48 hr, the virus-containing medium was filtered (0.45 μm filter, Millipore) and supplemented with 4 μg/ml polybrene (Sigma) (first supernatant). Viruses were collected for an additional 8 hr as before (second supernatant). Target fibroblasts were plated at 8×10^5 cells per 10 cm dish and incubated overnight. For infections, the culture medium was replaced by the appropriate first supernatant, and then the culture plates were centrifuged (1 hr, 1500 rpm) and incubated at 37°C for 8 hr. The infection process was repeated using the second supernatant. One plate of Phoenix cells produced sufficient virus for one to two plates of target cells. Sixteen hours later, infected cell populations were purified using the appropriate selection: 2 μg/ml puromycin; 75 μg/ml hygromycin; or 200 μg/ml or 400 μg/ml G418 (IMR90 and REF52 cells, respectively). Cells infected with WZL-CD8-based vectors were gently trypsinized and purified using magnetic beads conjugated to anti-CD8 (Dynal). Multiple genes were introduced sequentially, with the appropriate drug selection between. The ecotropic retrovirus receptor was introduced into human and rat fibroblasts (IMR90, WI38, and REF52) by first infecting cell populations with an amphotropic vector (pWZL-Neo-EcoR [a gift of D. Conklin] produced in BING cells), allowing subsequent infection with ecotropic viruses.

Growth Curves

Twenty-five thousand cells per well were plated into 12-well plates. At the indicated times, cells were washed with PBS, fixed in 10% formalin, and rinsed with distilled water. Cells were stained with 0.1% crystal violet (Sigma) for 30 min, rinsed extensively, and dried. Cell-associated dye was extracted with 2.0 ml 10% acetic acid. Aliquots were diluted 1:4 with H_2O, transferred to 96-well microtiter plates, and the optical density at 590 nm was determined. Values were normalized to the optical density at day 0 for the appropriate cell type. Within an experiment, each point was determined in triplicate; each growth curve was performed at least twice.

Cell-Cycle Analysis

Subconfluent cultures were labeled for 4 hr with 10 μM bromodeoxyuridine (BrdU; Amersham). Cells were detached with trypsin, fixed in 50% ethanol, and treated as follows (PBS washes between each step): 0.1 mg/ml of RNase A for 30 min at 37°C; 2 M HCl, 0.5% Triton X-100 for 30 min at room temperature; FITC-conjugated anti-BrdU (Becton Dickinson) diluted 1:5 for 1 hr at room temperature; and, finally, 5 μg/ml propidium iodide, 0.1 mg/ml RNAse A, 0.1% NP-40, and 0.1% trisodium citrate. Samples were analyzed by two-dimensional flow cytometry to detect both fluorescein and propidium iodide. For cytological analysis, cells were plated on coverslips, labeled with BrdU as above, and fixed in 5% acetic acid, 95% ethanol (15 min at −20°C). Positive nuclei were visualized using the Amersham cell proliferation kit, except that BrdU was detected with an FITC-conjugated secondary antibody (CalBiochem). Cells were counterstained with DAPI to identify all nuclei, and the percentage of BrdU-labeled cells (FITC/DAPI) was quantified using a fluorescence microscope. At least 200 cells were counted per sample; each experiment was performed at least twice.

Immunoblot Analysis

Cells were washed with ice-cold PBS and lysed in NP-40 lysis buffer (150 mM NaCl, 1.0% NP-40, 50 mM Tris–HCl [pH 8.0], 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, 1 mM sodium vanadate, 1 mM EDTA). After 30 min on ice, lysates were cleared by centrifugation. Samples corresponding to 15 μg of protein (BIO–RAD protein assay) were separated on 10% or 15% SDS–PAGE gels and transferred to Immobilon-P membranes (Millipore). Western blot analysis was accomplished according to standard procedures using ECL detection (Amersham). The following primary antibodies were used (1:1000 unless otherwise indicated): CM5 and CM1 (Novacastra) for human and rodent p53, respectively; 1 μg/ml WAF1 Ab1 (Oncogene Sciences) for human p21; an affinity-purified rabbit polyclonal antibody (Brugarolas et al., 1995) for rodent p21; DCS-50 (Novocastra) for human p16; M-156 (Santa Cruz) for mouse p16; a mixture of 0.5 μg/ml G3–245 mouse antibody (Pharmingen) and 1:100 dilutions of the C-36 and XZ-55 hybridoma culture supernatants; and a rabbit polyclonal antiserum against GST-human cyclin A for human and rodent cyclin A. Horseradish peroxidase-conjugated donkey anti-rabbit or sheep anti-mouse antibodies (Amersham) were used as secondary antibodies.

CDK2 Kinase Assays

Anti-CDK2 immunoprecipitates (Xiong et al., 1993) were washed two additional times with kinase buffer (20 mM Tris–HCl [pH 8.0], 10 mM $MgCl_2$, 1 mM EDTA, 1 mM DTT), and bead suspensions (∼10 μl) were brought to 20 μl with a solution containing 2.5 μg of histone H1 (Boehringer) and 10 μCi of [γ-^{32}P]ATP (3000 Ci/mmol). Reaction mixtures were incubated for 15 min at room temperature and resolved on 10% SDS–PAGE gels. H1 kinase activity was quantified using a Fuji Phosphorimager.

Northern Blots

Total RNA was isolated from subconfluent cultures (Trizol, GIBCO–BRL). Ten micrograms was resolved by electrophoresis, transferred to Hybond-N+ membranes, and probed according to standard procedures. The 1 kb Pst1 fragment from v-*fos* (kindly provided by R. Maestro and S. Rizzo) was used to assess c-*fos* expression. The DNA probe for human *PAI-1* was purchased from Genome Systems (clone 268918; locus N35838).

Senescence-Associated β-Galactosidase

SA-β-gal activity was detected as previously described (Dimri et al., 1995) with slight modifications. Cells were washed once with PBS (pH 7.2), fixed with 0.5% glutaraldehyde (PBS [pH 7.2]), and washed in PBS (pH 7.2) supplemented with 1 mM $MgCl_2$. Cells were stained in X-gal solution (1 mg/ml X-gal [Boehringer], 0.12 mM $K_3Fe[CN]_6$, 0.12 mM $K_4Fe[CN]_6$, 1 mM $MgCl_2$ in PBS at pH 6.0) overnight at 37°C.

Acknowledgments

We are sincerely indebted to H. E. Ruley for his early insights about this project. We acknowledge P. Burfeind, D. Horowitz, R. Maestro, J. Morgenstern, G. P. Nolan, and S. Rizzo for help, reagents, and assistance, and we thank D. Conklin and K. Galaktionov for critical reading of the manuscript. M. S. was supported by a Special Fellowship from the Leukemia Society of America. D. B. is an Investigator of the Howard Hughes Medical Institute and is supported by grant CA68040 from the NIH. S. W. L. is supported by PHS grant CA13106 from the NCI.

Received December 13, 1996; revised January 29, 1997.

References

Afshari, C.A., Vojta, P.J., Annab, L.A., Futreal, P.A., Willard, T.B., and Barrett, J.C. (1993). Investigation of the role of G1/S cell cycle mediators in cellular senescence. Exp. Cell Res. *209*, 231–237.

Alcorta, D.A., Xiong, Y., Phelps, D., Hannon, G., Beach, D., and Barrett, J.C. (1996). Involvement of the cyclin-dependent kinase inhibitor p16 (INK4a) in replicative senescence of normal human fibroblasts. Proc. Natl. Acad. Sci. USA *93*, 13742–13747.

Atadja, P., Wong, H., Garkavtsev, I., Veillette, C., and Riabowol, K. (1995). Increased activity of p53 in senescing fibroblasts. Proc. Natl. Acad. Sci. USA *92*, 8348–8352.

Bar-Sagi, D., and Feramisco, J.R. (1985). Microinjection of the *ras* oncogene protein into PC12 cells induces morphological differentiation. Cell *42*, 841–848.

Barbacid, M. (1987). *ras* genes. Annu. Rev. Biochem. *56*, 779–827.

Bartkova, J., Lukas, J., Guldberg, G., Alsner, J., Kirkin, A.F., Zuethen, J., and Bartek, J. (1996). The p16-cyclin D/Cdk4-pRb pathway as a functional unit frequently altered in melanoma pathogenesis. Cancer Res. *56*, 5475–5483.

Benito, M., Porras, A., Nebreda, A.R., and Santos, E. (1991). Differentiation of 3T3-L1 fibroblasts to adipocytes induced by transfection of *ras* oncogenes. Science *253*, 565–568.

Bos, J.L. (1988). The ras gene family and human carcinogenesis. Mutat. Res. *195*, 255–271.

Brugarolas, J., Chandrasekaran, C., Gordon, J.I., Beach, D., Jacks, T., and Hannon, G. (1995). Radiation-induced cell cycle arrest compromised by p21 deficiency. Nature *377*, 552–557.

Cahill, M.A., Janknecht, R., and Nordheim, A. (1996). Signalling pathways: jack of all cascades. Curr. Biol. *6*, 16–19.

Caldas, C., Hahn, S., da Costa, L.T., Redston, M.S., Schutte, M., Seymour, A.B., Weinstein, C.L., Hruban, R.H., Yeo, C.J., and Kern, S.E. (1994). Frequent somatic mutations and homozygous deletions of the p16 (*MTS1*) gene in pancreatic adenocarcinoma. Nature Genet. *8*, 27–32.

Dimri, G.P., Lee. X., Basile, G., Acosta, M., Scott, G., Roskelley, C., Medrano, E.E., Linskens, M., Rubelj, I., Pereira-Smith, O., Peacocke, M., and Campisi, J. (1995). A biomarker that identifies senescent human cells in culture and in aging skin in vivo. Proc. Natl. Acad. Sci. USA *92*, 9363–9367.

Donehower, L.A., Harvey, M., Slagle, B.L., McArthur, M.J., Montgomery, C.A., Jr., Butel, J.S., and Bradley, A. (1992). Mice deficient for p53 are developmentally normal but susceptible to spontaneous tumours. Nature *356*, 215–221.

Dulic, V., Drullinger, L.F., Lees, E., Reed, S.I., and Stein, G.H. (1993). Altered regulation of G1 in senescent human diploid fibroblasts: accumulation of inactive cyclin E-Cdk2 and cyclin D1-Cdk2 complexes. Proc. Natl. Acad. Sci. USA *90*, 11034–11038.

Fairweather, D.S., Fox, M., and Margison, G.P. (1987). The in vitro lifespan of MRC-5 cells is shortened by 5-azacytidine-induced demethylation. Exp. Cell Res. *168*, 153–159.

Franza, B.R., Jr., Maruyama, K., Garrels, J.I., and Ruley, H.E. (1986). In vitro establishment is not a sufficient prerequisite for transformation by activated Ras oncogenes. Cell *44*, 409–418.

Galaktionov, K., Lee, A.K., Eckstein, E., Draetta, G., Mecklet, J., Loda, M., and Beach, D. (1995). CDC25 phosphatases as potential human oncogenes. Science *269*, 1575–1577.

Galaktionov, K., Chen, X., and Beach, D. (1996). Cdc25 cell-cycle phosphatase as a target of c-*myc*. Nature *382*, 511–517.

Goldstein, S., Moerman, E.J., Fujii, S., and Sobel, B.E. (1994). Overexpression of plasminogen activator inhibitor type-1 in senescent fibroblasts from normal subjects and those with Werner syndrome. J. Cell Physiol. *161*, 571–579.

Hara, E., Smith, R., Parry, D., Tahara, H., Stone, S. and Peters, G. (1996). Regulation of p16^{CDKN2} expression and its implications for cell immortalization and senescence. Mol. Cell Biol. *16*, 859–867.

Harvey, D.M., and Levine, A.J. (1991). p53 alteration is a common event in the spontaneous immortalization of primary BALB/c murine embryo fibroblasts. Genes Dev. *5*, 2357–2385.

Harvey, M., Sands, A.T., Weiss, R.S., Hegi, M.E., Wiseman, R.W., Pantazis, P., Giovanella, B.C., Tainsky, M.A., Bradley, A., and Donehower, L.A. (1993). *In vitro* growth characteristics of embryo fibroblasts isolated from p53-deficient mice. Oncogene *8*, 2457–2467.

Hayflick, L., and Moorhead, P.S. (1961). The serial cultivation of human diploid cell strains. Exp. Cell Res. *25*, 585–621.

Hicks, G.G., Egan, S.E., Greenberg, A.H., and Mowat, M. (1991). Mutant p53 tumor suppressor alleles release *ras*-induced cell cycle growth arrest. Mol. Cell Biol. *11*, 1344–1352.

Hinds, P.W., Dowdy, S.F., Eaton, E.N., Arnold, A., and Weinberg, R.A. (1994). Function of a human cyclin gene as an oncogene. Proc. Natl. Acad. Sci. USA *91*, 709–713.

Hirakawa, T., and Ruley, H.E. (1988). Rescue of cells from *ras* oncogene-induced growth arrest by a second complementing oncogene. Proc. Natl. Acad. Sci. USA *85*, 1519–1523.

Hirama, T., and Koeffler, H.P. (1995). Role of the cyclin-dependent kinase inhibitors in the development of cancer. Blood *86*, 841–854.

Holliday, R. (1986). Strong effects of 5-azacytidine on the in vitro lifespan of human diploid fibroblasts. Exp. Cell Res. *166*, 543–552.

Hollstein, M., Shomer, B., Greenblatt, M., Soussi, T., Hovig, E., Montesano, R., and Harris, C.C. (1996). Somatic point mutations in the p53 gene of human tumors and cell lines: updated compilation. Nucleic Acids Res. *24*, 141–146.

Hunter, T. (1991). Cooperation between oncogenes. Cell *64*, 249–270.

Jacks, T., Remington, L., Williams, B.O., Schmitt, E.M., Halachmi, S., Bronson, R.T., and Weinberg, R.A. (1994). Tumor spectrum analysis in p53-mutant mice. Curr. Biol. *4*, 1–7.

Kemp, C.J., Donehower, L.A., Bradley, A., and Balmain, A. (1993). Reduction of p53 gene dosage does not increase initiation or promotion but enhances malignant progression of chemically induced skin tumors. Cell *74*, 813–822.

Kinzler, K.W., and Vogelstein, B. (1996). Lessons from hereditary colorectal cancer. Cell *87*, 159–170.

Ko, L.J., and Prives, C. (1996). p53: puzzle and paradigm. Genes Dev. *10*, 1054–1072.

Land, H., Parada, L.F., and Weinberg, R.A. (1983). Tumorigenic conversion of primary embryo fibroblasts requires at least two cooperating oncogenes. Nature *304*, 596–602.

Linardopoulos, S., Street, A., Quelle, D.E., Parry, D., Peters, G., Sherr, C.J., and Balmain, A. (1995). Deletion and altered regulation of *p16*^{INK4a} and *p15*^{INK4b} in undifferentiated mouse skin tumors. Cancer Res. *55*, 5168–5172.

Liu, J.J., Chao, J.R., Jiang, M.C., Ng, S.Y., Yen, J.J., and Yang-Yen, H.F. (1995). Ras transformation results in an elevated level of cyclin D1 and acceleration of G1 progression in NIH 3T3 cells. Mol. Cell Biol. *15*, 3654–3663.

Lovec, H., Sewing, A., Lucibello, F.C., Müller, R., and Möröy, T. (1994). Oncogenic activity of cyclin D1 revealed through cooperation with Ha-ras: link between cell cycle control and malignant transformation. Oncogene *9*, 323–326.

Lowe, S.W., and Ruley, H.E. (1993). Stabilization of the p53 tumor suppressor is induced by adenovirus 5 E1A and accompanies apoptosis. Genes Dev. *7*, 535–545.

Lowe, S.W., Ruley, H.E., Jacks, T., and Housman, D.E. (1993). p53-dependent apoptosis modulates the cytotoxicity of anticancer agents. Cell *74*, 957–967.

Lowe, S.W., Jacks, T., Housman, D.E., and Ruley, H.E. (1994). Abrogation of oncogene-associated apoptosis allows transformation of p53-deficient cells. Proc. Natl. Acad. Sci. USA *91*, 2026–2030.

Lumpkin, C.K., Knepper, J.E., Butel, J.S., Smith, J.R., and Pereira-Smith, O.M. (1986). Mitogenic effects of the proto-oncogene and oncogene forms of c-Ha-ras DNA in human diploid fibroblasts. Mol. Cell Biol. *6*, 2990–2993.

Marshall, C.J. (1995). Specificity of receptor tyrosine kinase signaling: transient versus sustained extracellular signal-regulated kinase activation. Cell *80*, 179–185.

McCurrach, M.E., Connor, T.M.F., Knudson, C.M., Korsmeyer, S.J., and Lowe, S.W. (1997). *bax*-deficiency promotes drug resistance and oncogenic transformation by attenuating p53-dependent apoptosis. Proc. Natl. Acad. Sci. USA, in press.

Metz, T., Harris, A.W., and Adams, J.M. (1995). Absence of p53 allows direct immortalization of hematopoietic cells by the *myc* and *raf* oncogenes. Cell *82*, 29–36.

Morgenstern, J.P., and Land, H. (1990). Advanced mammalian gene

transfer: high titre retroviral vectors with multiple drug selection markers and a complementary helper-free packaging cell line. Nucleic Acids Res. *18*, 3587–3596.

Mu, X.-C., and Higgins, P.J. (1995). Differential growth state-dependent regulation of plasminogen activator inhibitor type-1 expression in senescent IMR-90 human diploid fibroblasts. J. Cell. Physiol. *165*, 647–657.

Newbold, R.F., and Overell, R.W. (1983). Fibroblast immortality is a prerequisite for transformation by EJ c-H-*ras* oncogene. Nature *304*, 648–651.

Noda, A., Ning, Y., Venable, S.F., Pereira-Smith, O.M., and Smith, J.R. (1994). Cloning of senescent cell-derived inhibitors of DNA synthesis using an expression screen. Exp. Cell Res. *211*, 90–98.

Ogryzko, V.V., Hirai, T.H., Russanova, V.R., Barbie, D.A., and Howard, B.H. (1996). Human fibroblast commitment to a senescence-like state in response to histone deacetylase inhibitors is cell cycle dependent. Mol. Cell Biol. *16*, 5210–5218.

Quelle, D.E., Ashmun, R.E., Hannon, G.J., Rehberger, P.A., Trono, D., Richter, K.H., Walker, C., Beach, D., Sherr, C.J., and Serrano, M. (1995). Cloning and characterization of murine $p16^{INK4a}$ and $p15^{INK4b}$ genes. Oncogene *11*, 635–645.

Reznikoff, C.A., Yeager, T.R., Belair, C.D., Savelieva, E., Puthenveettil, J.A., and Stadler, W.M. (1996). Elevated p16 at senescence and loss of p16 at immortalization in human papillomavirus 16 E6, but not E7, transformed human uroepithelial cells. Cancer Res. *56*, 2886–2890.

Ridley, A.J., Paterson, H.F., Noble, M., and Land, H. (1988). *ras*-mediated cell cycle arrest is altered by nuclear oncogenes to induce Schwann cell transformation. EMBO J. *7*, 1635–1645.

Rogan, E.M., Bryan, T.M., Hukku, B., Maclean, K., Chang, A.C.-M., Moy, E.L., Englezou, A., Warneford, S.G., Dalla-Pozza, L., and Reddel, R.R. (1996). Alterations in p53 and $p16^{INK4}$ expression and telomere length during spontaneous immortalization of Li-Fraumeni syndrome fibroblasts. Mol. Cell Biol. *15*, 4745–4753.

Röhme, D. (1981). Evidence for a relationship between longevity of mammalian species and life spans of normal fibroblasts in vitro and erythrocytes in vivo. Proc. Natl. Acad. Sci. USA *78*, 5009–5013.

Ruley, H.E. (1983). Adenovirus early region 1A enables viral and cellular transforming genes to transform primary cells in culture. Nature *304*, 602–606.

Ruley, H.E. (1990). Transforming collaborations between *ras* and nuclear oncogenes. Cancer Cells *2*, 258–268.

Serrano, M., Hannon, G.J., and Beach, D. (1993). A new regulatory motif in cell-cycle control causing specific inhibition of cyclin D/CDK4. Nature *366*, 704–707.

Serrano, M., Gómez-Lahoz, E., DePinho, R.A., Beach, D., and Bar-Sagi, D. (1995). Inhibition of ras-induced proliferation and cellular transformation by $p16^{INK4}$. Science *267*, 249–252.

Serrano, M., Lee, H.-W., Chin, L., Cordon-Cardo, C., Beach, D., and DePinho, R.A. (1996). Role of the *INK4a* locus in tumor suppression and cell mortality. Cell *85*, 27–37.

Seshadri, T., and Campisi, J. (1990). Repression of c-*fos* transcription and an altered genetic program in senescent human fibroblasts. Science *247*, 205–209.

Sherr, C.J., and Roberts, J.M. (1995). Inhibitors of mammalian G1 cyclin-dependent kinases. Genes Dev. *9*, 1149–1163.

Stein, G.H., and Dulic, V. (1995). Originis of G1 arrest in senescent human fibroblasts. Bioessays *17*, 537–543.

Sullivan, N.F., Sweet, R.W., Rosenberg, M., and Feramisco, J.R. (1986). Microinjection of the *ras* oncogene protein into nonestablished rat embryo fibroblasts. Cancer Res. *46*, 6427–6432.

Suzuki, T., Kitao, S., Matsushime, H., and Yoshida, M. (1996). HTLV-1 Tax protein interacts with cyclin-dependent kinase inhibitor $p16^{INK4A}$ and counteracts its inhibitory activity towards CDK4. EMBO J. *15*, 1607–1614.

Tanaka, N., Ishihara, M., Kitagawa, M., Harada, H., Kimura, T., Matsuyama, T., Lamphier, M.S., Aizawa, S., Mak, T.W., and Taniguchi, T. (1994). Cellular commitment to oncogene-induced transformation or apoptosis is dependent on the transcription factor IRF-1. Cell *77*, 829–839.

Venable, M.E., Lee, J.Y., Smyth, M.J., Bielawska, A., and Obeid, L.M. (1995). Role of ceramide in cellular senescence. J. Biol. Chem. *270*, 30701–30708.

Vogelstein, B., and Kinzler, K.W. (1993). The multistep nature of cancer. Trends Genet. *9*, 138–141.

Votja, P.J., and Barrett, J.C. (1995). Genetic analysis of cellular senescence. Biochim. Biophys. Acta *1242*, 29–41.

Weinberg, R.A. (1989). Oncogenes, antioncogenes, and the molecular bases of multistep carcinogenesis. Cancer Res. *49*, 3713–3721.

Weinberg, R.A. (1995). The retinoblastoma protein and cell cycle control. Cell *81*, 323–330.

Wölfel, T., Hauer, M., Schneider, J., Serrano, M., Wölfel, C., Klehmann-Hieb, E., De Plaen, E., Hankeln, T., Meyer zum Büschenfelde, K.-H., and Beach, D. (1995). A $p16^{INK4a}$-insensitive CDK4 mutant targeted by cytolytic T lymphocytes in a human melanoma. Science *269*, 1281–1284.

Xiong, Y., Zhang, H., and Beach, D. (1993). Subunit rearrangement of the cyclin-dependent kinases is associated with cellular transformation. Genes Dev. *7*, 1572–1583.