Inactivation of p16INK4a, with Retention of pRB and p53/p21cip1 Function, in Human MRC5 Fibroblasts That Overcome a Telomere-independent Crisis during Immortalization*

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Recent investigations, including our own, have shown that specific strains of fibroblasts expressing telomerase reverse transcriptase (hTERT) have an extended lifespan, but are not immortal. We previously demonstrated that hTERT-transduced MRC5 fetal lung fibroblasts (MRC5hTERTs) bypassed senescence but eventually succumbed to a second mortality barrier (crisis). In the present study, 67 MRC5hTERT clones were established by limiting dilution of a mass culture. Whereas 39/67 clones had an extended lifespan, 39 extended lifespan clones underwent crisis. 11 of 39 clones escaped crisis and were immortalized. There was no apparent relationship between the fate of clones at crisis and the level of telomerase activity. Telomeres were hyperextended in the majority of the clones analyzed. There was no difference in telomere length of pre-crisis compared with post-crisis and immortal clones, indicating that hyperextended telomeres were conducive for immortalization and confirming that crisis was independent of telomere length. Immortalization of MRC5hTERT cells was associated with repression of the cyclin-dependent kinase inhibitor p16INK4a and up-regulation of pRB. However, the regulation of pRB phosphorylation and the response of the p55/p21cip1/p16INK4a pathway were normal in immortal cells subject to genotoxic stress. Overexpression of oncogenic ras failed to de-repress p16INK4a in immortal cells. Furthermore, expression of ras enforced senescent-like growth arrest in p16INK4apositive, but not p16INK4anegative MRC5hTERT cells. Immortal cells expressing ras formed small, infrequent colonies in soft agarose, but were non-tumorigenic. Overall, these results implicate the inactivation of p16INK4a as a critical event for overcoming telomere-independent crisis, immortalizing MRC5 fibroblasts and overcoming ras-induced premature senescence.

One characteristic that is common to most (if not all) cancer cells and distinct from normal somatic cells is that cancer cells proliferate indefinitely (they are immortal), whereas the replicative lifespan of normal cells is limited and tightly regulated. The mechanisms that limit the replicative lifespan of normal human somatic cells are therefore thought to function to prevent immortalization and carcinogenesis (1). When normal somatic cells reach the end of their replicative lifespan, they undergo an irreversible growth arrest, referred to as senescence. Replicative senescent cells arrest in G1 phase of the cell cycle and express high levels of the cyclin-dependent kinase (CDK) inhibitors (CDKIs), p16INK4a and p21cip1 (2, 3). Further, overexpression of p21cip1, p16INK4a, or p53 (which transcriptionally induces p21cip1) arrests human cells in a senescent-like state in the G1 phase of the cell cycle (4). Increased expression of p21cip1 inhibits the activity of CDK2-cyclin E complexes, as well as other CDK/cyclin complexes, while p16INK4a specifically sequesters and inactivates CDK4/6 complexes (5). Inactivation of these cyclin-CDK complexes prevents phosphorylation of the retinoblastoma protein (pRB), which is necessary for progression from G1 to S phase of the cell cycle. Hence, accumulation of p16INK4a and p21cip1 appear to be critical mediators of growth arrest in senescent cells. Consistent with this possibility, inactivation of p53, pRB, or p21cip1 was shown to enable normal human cells to proliferate beyond senescence (6–8). However, the vast majority of cells that bypass senescence as a consequence of inactivation of these pathways are not immortal, as they ultimately succumb to a second proliferative barrier, called crisis (9).

Compelling evidence implicates the gradual shortening of telomeres as an upstream event in the initiation of CDK inhibitor activity and induction of senescence. Telomeres are specialized chromosomal-end structures that prevent abnormal chromosomal fusions and rearrangements (10). In normal human cells telomeres shorten with each cell division. When telomeres become critically short, chromosomal instability ensues and p53-dependent DNA damage signals initiate the senescence program. Senescence may also be triggered by telomere-independent stresses, such as DNA damage caused by irradiation and cytotoxic drugs or chemically induced oxidative stress (11). In addition, premature senescence may be induced by expression of oncogenic ras and ras, which signal via the mitogen-activated kinase cascade to induce expression of p16INK4a and p53 (12–14).

In contrast to telomere attrition observed in normal somatic
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cells, ~80–90% of cancer cells and immortal cell lines have telomeres stabilized or elongated by the enzyme telomerase (15). Telomerase is a ribonuclear protein complex that has a reverse transcriptase (hTERT) as a catalytic domain (16). Ectopic expression of hTERT was shown to be sufficient to reconstitute telomerase enzyme activity, elongate telomeres, and extend the lifespan of normal human fibroblasts and retinal epithelial cells (17–20). In addition, it was reported that expression of hTERT immortalized specific strains of fibroblasts, epithelial, and endothelial cells, without the requirement for molecular alterations in p53/p21<sup>WAF1</sup> and pRB/p16INK4a pathways, and without phenotypic changes associated with carcinogenesis (21–23). However, other investigators have found reconstitution of telomerase enzyme activity to be insufficient for immortalization keratinocytes, mammary, and adenoid epithelial cells, as well as bone marrow, brain, and mammary-derived endothelial cells (24–29). In the case of mammary epithelial cells and keratinocytes, inactivation of p16INK4a expression was necessary for hTERT-transduced cells to proliferate beyond senescence (24). The apparent inability of hTERT to immortalize specific cell types was proposed to be caused by inadequate culture conditions, which induced p16INK4a expression (30). However, more recent investigations found no difference in the replicative capacity of hTERT-transduced keratinocytes and bone marrow endothelial cells cultured under alternative culture conditions (29, 31). Overall, these results suggest that a telomere-independent barrier may operate to prevent immortalization of some strains of human epithelial and endothelial cells.

There is also a growing body of evidence indicating that expression of catalytically active telomerase is insufficient for immortalization of some strains of human fibroblasts. Our previous study, as well as independent investigations, demonstrated that several different stains of fibroblasts expressing hTERT ceased proliferating after a significantly extended lifespan (32–35). In our study of hTERT-transduced MRC5 lung fibroblasts (MRC5hTERT), growth arrest was not permanent, as subsets of mass cultured cells eventually resumed proliferation, and immortal cell lines were established. Similarly, Noble et al. (35) have recently shown that hTERT-transduced human foreskin fibroblasts undergo a period of reduced growth during immortalization. The growth lags observed in hTERT-transduced fibroblast cultures are consistent with the requirement for co-operating molecular events for immortalization. During the period of delayed growth, MRC5hTERT cells exhibited features that were characteristic of cells driven beyond senescence and into crisis by viral oncogenes, which inactivate the p53 and pRB tumor suppressor pathways (6, 9, 36, 37). Substantial evidence indicates that crisis in viral oncogene transformed cells is a consequence of critical telomere shortening (38–40). However, the growth barrier observed in hTERT-transduced, telomerase positive cultures provides evidence for a telomere-independent crisis. While crisis in oncogene transformed cells has been extensively investigated, hTERT-driven crisis remains largely uncharacterized.

In the present study, 67 MRC5hTERT clones were established to enable investigation of hTERT-driven crisis. Our results demonstrate that all MRC5hTERT clones that had an extended lifespan were subject to a telomere-independent crisis, while only 28% of lifespan extended clones escaped crisis and were immortalized. Immortalization was associated with inactivation of p16INK4a, which was not reversed by expression of oncogenic ras. However, the regulation of pRB phosphorylation and function of the p53/p21<sup>WAF1</sup> pathway was normal in immortal cells. Our results indicate that inactivation of p16INK4a may be necessary to overcome telomere-independent crisis during immortalization of MRC5 fibroblasts.

EXPERIMENTAL PROCEDURES

Cell Culture—MRC-5 human fetal lung fibroblasts were purchased from the American Type Culture Collection (Manassas, VA). MRC5hTERT-1 were previously established by retroviral transduction of MRC5 cells with retroviral vector TiG, which encodes hTERT upstream of an internal ribosomal entry site (IRES) and a cDNA encoding the green fluorescence protein (GFP) (32). MRC5hTERT-1 was referred to as MRC5hTERT-1 in our previous report. Except where otherwise indicated, MRC5 and MRC5hTERT-1 cells were grown in α-Minimal Essential Medium (αMEM) with 2 mM t-glutamine, 100 units/ml penicillin and 100 μg/ml streptomycin (Invitrogen Life Technologies) plus 10% fetal bovine serum (FBS) (Trace Scientific, VIC, Australia) at 37°C/5% CO2. MRC5hTERT clones were established by plating the MRC5hTERT-1 mass cultured cells at 66 population doublings (PDs) at a concentration of 20 cells/ml in a 96-well plate and identifying wells containing single cells under an inverted microscope. For colony formation in soft agarose, cells were plated at 10,000/ml in 0.33% agarose (Seaplaque, Cambrex, Rockland, ME)/20% FBS/αMEM over a pre-formed layer of 0.5% agarose/20% FBS/αMEM and incubated at 37°C/5% CO2. Colonies of greater than 50 cells each were scored under an inverted microscope on day 14.

Telomerase Assay—Telomerase expression was quantified using the PCR-based telomeric repeat amplification protocol (TRAP) as described previously (15). Protein was extracted by lysing cells in CHAPS lysis buffer (10 mM Tris, pH 7.5, 1 mM MgCl2, 1 mM EDTA, 0.1 mM benzamidine, 5 mM β-mercaptoethanol, 0.5% CHAPS, 10% glycerol) on ice for 30 min. Supernatant was collected following centrifugation at 12,000 × g for 30 min at 4°C. Aliquots of 2 μg of protein were assayed in 25-μl reactions, which included TS primer, NT primer, and 0.01 amol of internal PCR control (TSNT). Each TRAP assay included a negative control (CHAPS buffer), a positive control (protein extract from the SK-N-SH neuroblastoma cell line) and the R8 quantification oligonucleotide. PCR products were purified through a 12.5% polyacrylamide gel and visualized with 0.01% (v/v) SYBR® Green 1 nucleic acid gel stain (Molecular Probes, St. Louis, MI). Images were captured by laser scanning using a Typhoon 9410 Variable Mode Imager (Molecular Dynamics, Sunnyvale, CA) and analyzed using ImageQuant (Molecular Dynamics). Telomerase activity in each sample, or total product generated (TGP) was calculated as a fraction of TGP in the neuroblastoma control: TGP = 100 × [CT − B]/TSNT/[NB − B]/TSNTNB, where abbreviations are as follows; T is sample signal, B is background of negative control, TSNT is the internal control signal, NB is the neuroblastoma signal, and TSNTNB is the signal of the internal control band of the neuroblastoma control.

Telomeric Restriction Fragment (TRF) Assay—Genomic DNA was extracted by standard phenol/chloroform procedure and precipitated with 0.3 M sodium acetate and 2 volumes of ethanol. For TRF assays, 10 μg of DNA was digested with HinfI and RsaI restriction enzymes (New England Biolabs, Beverly, MA) for 16 h at 37°C, followed by electrophoresis through a 0.7% agarose gel. Gels were depurinated, denatured, neutralized, and transferred to Hybond-N+ nylon membrane (Amer sham Biosciences). Transfer was by capillary action using 20× SSC for 16 h, before fixing the transferred DNA to the membrane by UV cross-linking (120 mJ) (Stratagene, La Jolla, CA). Chemiluminescence detection of telomeric restriction fragments was performed using the TelTAGGG Telomere Length Assay (Roche Applied Science). In brief, the membrane was blocked 30 min at 42°C and hybridized with a digoxigenin (DIG)-labeled telomere probe. Further washes were performed according to the manufacturer's instruction before incubating with an anti-DIG alkaline phosphatase secondary antibody for 30 min at room temperature. CDP-star substrate (Roche Applied Science) was added for detection of teleomers, and the membrane was exposed to x-ray film (Fuji Film, Japan). Images were scanned on a flat bed scanner and TRF signals quantified using MacBas (Fuji). Mean TRF lengths were determined according to the following formula: [S/LS]/[Ss], where abbreviations are as follows; Ss is TRF signal at a given location after background subtraction, S is the corresponding length at position i (41).

Western Blot Analysis—Protein lysates were collected from cell pellets by incubation on ice in 10 mM Tris, pH 8.0, 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 1% protease inhibitor (Sigma) for 30 min. Protein concentrations were determined with the BCA assay (Pierce). For detection of p14<sup>INK4a</sup>, p21<sup>CIP1</sup>, pRB, and total actin expression, aliquots of 25 μg of protein were separated by SDS-PAGE through a
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15% polyacrylamide gel and transferred to polyvinylidine difluoride (PVDF) membrane (Millipore, Bedford, MA). For detection of pRB phosphorylation status, 50 μg of protein was separated on 7%, polyacrylamide gel and transferred to PVDF membrane. Membranes were stained with 0.1% (w/v) Ponceau S (Sigma) in 5% aqueous acetic acid to confirm even transfer of proteins, then destained in demineralized water. Membranes were blocked in 5% skim milk and hybridized to the following primary antibodies: mouse anti-human p21\(^{\text{wt}}\) monoclonal antibody (BD Pharmingen, San Diego, CA) 1/5000, rabbit anti-human p16\(^{\text{INK4a}}\) polyclonal antibody (Biosource, Nivelles, Belgium) 1/1000, rabbit anti-human actin 1/50000, mouse anti-human RB antibody (BD Biosciences, San Jose, CA) 1/5000. Membranes were washed in 20 mM Tris, pH 7.5, 150 mM NaCl, 0.05% (v/v) Tween-20, and hybridized to the corresponding horseradish peroxidase-conjugated secondary antibody; anti-rabbit (Amersham Biosciences) 1:10000 or anti-mouse (Amersham Biosciences) 1:5000, for 1 h at room temperature. Chemiluminescence detection was performed using Supersignal (Pierce) according to the manufacturer’s instructions and images were acquired on x-ray film or Versdoc Imaging System (Bio-Rad).

Retroviral Transduction—Stable retroviral producers, which generate high titers of the replication defective retroviral vectors L9HGF and L9N1GFP, were previously established and generously provided by A/Prof. Geoff Symonds and Dr. Alla Dolnikov (Children’s Cancer Institute Australia for Medical Research) (42). The L9N1GFP vector encodes mutant N-\(\alpha\)-ras (N-\(\alpha\)-ras13, G-C transversion at position 763) and a yellow fluorescence protein reporter gene GFP/Fpz (referred to as YFP in this report) that are linked by an IRES. Expression of N-\(\alpha\)-ras protein was previously shown to correlate with YFP fluorescence (42). L9HGF, which encodes YFP alone, was used as a control. For simplicity, the L9HGF and L9N1GFP vectors are referred to as Nras-YFP and YFP vectors, respectively, in the present report. Retroviral vectors, encoding wild-type p16\(^{\text{ink4a}}\) (p16wt), p16\(^{\text{ink4a}}\) with an inactivating missense mutation (p16M53T) and the control vector, pBABEpuro, were generously provided by Dr. Gordon Peters (Imperial Cancer Research Fund Laboratories, London, UK) (43). Expression and function of p16wt and p16M53T were previously demonstrated in retrovirally transduced fibroblasts (43). In the present study, plasmid retroviral vectors were transfected into Phoenix A packaging cells (obtained from American Type Culture Collection with permission from Garry Nolan of Stanford University) using a standard calcium phosphate transfection procedure. MRC5 cells, and MRC5hTERT cells were transduced by overnight incubation in viral supernatant supplemented with 5 μg/ml polybrene (Sigma) for three consecutive days. Cells transduced with Nras-YFP and YFP were enumerated by flow cytometry using a FACs Caliber flow cytometer (BD Pharmingen). Cells transduced with p16wt, p16M53T, and pBABEpuro were selected by growth in 0.8 μg/ml puromycin (Sigma) in 10% αMEM.

β-Galactosidase Staining—Cells were assayed for senescence-associated β-galactosidase activity (SA-β-gal) at pH 6.0 or previously denatured. In brief, cells were fixed in 2% formaldehyde, 0.2% glutaraldehyde, and stained with 1 mg/ml 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-gal) (Sigma) for 12–16 h at 37°C. Cells expressing β-galactosidase activity (stained blue) were scored under an inverted microscope. At least three fields of 50–200 cells were scored for each sample assayed. Values are given as means with S.D.

RESULTS

MRC5hTERT Mass Cultures and Clones Succumb to Crisis—In our previous study, MRC5hTERT cells that were propagated as a mass culture underwent a growth crisis during immortalization (32). Crisis was reproducible and appeared to be programmed, as when the MRC5hTERT-1 mass culture was thawed at early passage, then propagated by a different operator, using a different media formulation and FBS from an alternate company, the growth pattern was identical (Fig. 1a).

To further investigate crisis, we established 67 MRC5hTERT clones by limiting dilution of the MRC5hTERT-1 culture at 66 PD. In our hands non-transduced MRC5 cells senesced at 62.5 ± 3.5 PD (32). Twenty-eight of the MRC5hTERT-1 clones that did not reach confluence in 24-well plates exhibited a senescent morphology. These clones were assumed to be telomerase-negative, non-transduced senescent MRC5 cells, although this could not be tested due to insufficient cell numbers. Since the transduction efficiency of the MRC5hTERT-1 mass culture was 77% (32), it would be expected that at least 23% of the clones would be non-transduced and therefore not bypass senescence. Thirty-nine clones achieved at least 80 PD and were considered to have an extended lifespan (Table I). However, all 39 extended lifespan clones eventually underwent crisis, which was characterized by a reduction in the expansion rate of the culture (Fig. 1b). Clones in crisis exhibited a senescent-like morphology and exhibited SA-β-gal activity (a hallmark of senescence), or underwent cell death and displayed morphologic features of apoptosis (Table I and Fig. 1c). 11 out of 39 lifespan-extended clones (28%) escaped crisis, continued to proliferate beyond 220 PD (which represented more than a 3-fold increase in lifespan) and were considered immortal (Fig. 1d). Two additional immortal clones (clones D1 and D2) were established by limiting dilution of the MRC5hTERT-1 mass culture at 396 PD. These clones were established to ensure that our analyses included clones that were most favored by the immortalization process.

Telomere Length and Telomerase Activity in MRC5hTERT Clones—To determine whether crisis was related to the level of telomerase activity, telomerase enzyme activity was measured in 10 representative clones using the TRAP assay (Fig. 2, a and b). For clones that escaped crisis and were immortalized, at least two time points, pre- and post-crisis, were assayed. To obtain quantitative data, each sample was run 3 times. Telomerase activity was detected in all MRC5hTERT samples analyzed, albeit at a range of levels. There was no clear association between the level of telomerase activity detected at early time points (PD 88–92) and the fate of the clone (senescent-like arrest or cell death at crisis). The level of telomerase activity in early passage clones appeared to be sufficient for immortalization, as there was no significant difference between the level of telomerase in pre-crisis (≤92 PD) versus post-crisis and immortal clones (≥141 PD) (Student’s t test, p = 0.9).

We previously determined that mean telomere length of non-transduced MRC5 cells shortened at a rate of 72 bp/PD (32). Assuming a constant rate of telomere shortening, we estimated that the telomere length of MRC5 cells was 8.1 Kbp at the time of transduction (26 PD). In the present study, 9/10 of the representative MRC5hTERT clones were found to have hyperextended telomeres (Fig. 2c). Prior to the onset of crisis, the mean TRF length of these nine clones ranged from 22–38 Kbp. There was very little change in telomere length between matched pre- and post-crisis samples of individual clones. Furthermore, telomeres remained hyperextended in post-crisis and immortal cells, indicating that the very long telomeres were unlikely to be the cause of crisis and hyperextended telomeres were conducive to immortalization. Clone 1 was an exception, with a mean TRF length of 6.0 Kbp at 89 PD, which reflects a telomere-shortening rate of 33 bp/PD. It is assumed that critically short telomeres were responsible for growth arrest of the latter clone at 98 PD. Significantly, clone 1 also expressed the lowest level of telomerase activity (Fig 2, a and b). Thus it appears that the low level of telomerase in clone 1 was below the threshold for telomere elongation, but reduced the rate of telomere shortening that would occur in absence of telomerase and thereby enabled a lifespan extension, but not immortalization.

Modulation of p16\(^{\text{INK4a}}\) and p21\(^{\text{cip1}}\) in MRC5hTERT Clones and Mass Culture—Western blot analysis was performed to investigate the expression of p16\(^{\text{INK4a}}\) and p21\(^{\text{cip1}}\) during immortalization of MRC5hTERT cells (Fig. 3). As expected, both p21\(^{\text{cip1}}\) and p16\(^{\text{INK4a}}\) were markedly elevated in senescent non-transduced MRC5 cells (PD 63) compared with earlier passage MRC5 cells (PD 41) (Fig. 3a, lanes 7 and 8). p16\(^{\text{INK4a}}\) accumulated in MRC5hTERT mass cultured cells to reach very high
levels at 97 PD, which was prior to crisis, and then returned to low levels by 124 PDs. In the Western blot shown in Fig. 3a, p16INK4a was undetectable in post-crisis cells at 154 and 260 PDs (Fig. 3a, lanes 5 and 6). However, further analyses, using an antibody from an alternate company (BIOSOURCE), revealed low levels of p16INK4a expression in post-crisis cells at 147, 159, and 181 PDs (data not shown). Irrespective of the antibody used, p16INK4a was undetectable at eight different time points between 203–420 PDs. Thus, although down-regulation of p16INK4a was detectable immediately after crisis, complete silencing of p16INK4a in MRC5hTERT mass culture did not correspond with escape from crisis, but appeared to occur between 181 and 203 PDs. Time course analyses of p21cip1 expression in MRC5hTERT mass cultured cells showed that p21cip1 was induced by 63 PD and reached levels comparable to senescent cells by 124 PD, when the culture exhibited dramatic cell death and slowed growth (Fig. 3a, lane 4). Expression of p21cip1 was reduced to low levels in post-crisis cells, but was not completely extinguished.

Expression of p16INK4a and p21cip1 was also assessed in representative MRC5hTERT clones (Fig. 3b). Both p16INK4a and p21cip1 were down-regulated in post-crisis cells compared with pre-crisis cells in two clones that underwent a transient period of cell death before becoming immortal (clones 2 and 12). A similar result was obtained for clone 49, which was subject to a short period of cell death prior to immortalization (data not shown). However, in contrast to clones that escaped crisis, p16INK4a was sustained at very high levels (comparable to senescent MRC5s) in clone 15 and clone 30, which arrested in a senescent like state (Fig. 3b and additional data not shown). Unexpectedly, expression of p21cip1 was lower in clone 15 at 140 PD, when cell proliferation halted, compared with an earlier time point (87 PD) (Fig. 3b). Similarly, very low levels of p21cip1 were detected in clone 30 at growth arrest (data not shown). Hence, repression of p21cip1 did not correlate with immortalization and was not sufficient for clones 15 and 30 to escape crisis. Sustained high-level p16INK4a was associated with, and therefore may have en-

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**Fig. 1.** Proliferation of MRC5hTERT cells. a, proliferation of MRC5hTERT-1 mass culture in 2 independent runs: CCIA, cells grown by A. James at Children’s Cancer Institute Australia for Medical Research (2001–2003), using gMEM plus 10% FBS (Trace Scientific). MSKCC, cells grown by K. L. MacKenzie at Memorial Sloan-Kettering Cancer Center (1998–2000), NY, in MEM with Earle’s balanced salt solution, 2 mM glutamine, and 10% FBS (Hyclone Logan, UT). b, proliferation of 39 MRC5hTERT clones that had an extended lifespan (proliferated beyond 80 PDs). Clones were established by limiting dilution of MRC5hTERT-1 culture at 66 PDs. c, photomicrographs of MRC5hTERT clone 15, which underwent permanent growth arrest at crisis and displayed morphologic features of senescence, and MRC5hTERT clone 12, which exhibited cell death at crisis, before eventually resuming proliferation and becoming immortal. White scale bar represents 50 μm. d, Kaplan-Meier curve showing survival of MRC5hTERT extended lifespan clones. Each point indicates the number of PD attained when a clone ceased growing, either because of cell death or senescent-like growth arrest.
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**TABLE I**

| Clone no.<sup>a</sup> | Fate at crisis<sup>b</sup> | X-gal positive<sup>c</sup> | Maximum PDs<sup>d</sup> |
|------------------------|--------------------------|--------------------------|------------------------|
| 1                      | Senescent                | 97                       | >220                   |
| 2                      | Cell death/immortalized  | n.d.<sup>e</sup>          | >220                   |
| 3                      | Senescent                | n.d.<sup>e</sup>          | 88                     |
| 4                      | Senescent                | n.d.<sup>e</sup>          | 87                     |
| 5                      | Senescent                | 72                       | 92                     |
| 6                      | Senescent                | n.d.<sup>e</sup>          | 106                    |
| 7                      | Senescent                | 100                      | 120                    |
| 8                      | Senescent                | 86                       | n.d.<sup>e</sup>       |
| 9                      | Cell death/immortalized  | n.d.<sup>e</sup>          | >220                   |
| 10                     | Senescent                | n.d.<sup>e</sup>          | 87                     |
| 11                     | Cell death/immortalized  | n.d.<sup>e</sup>          | >220                   |
| 12                     | Cell death/immortalized  | n.d.<sup>e</sup>          | >220                   |
| 13                     | Senescent/immortalized   | n.d.<sup>e</sup>          | >220                   |
| 14                     | Senescent                | n.d.<sup>e</sup>          | 140                    |
| 15                     | Senescent                | 93                       | 90                     |
| 16                     | Senescent                | 100                      | 92                     |
| 17                     | DD                      | 96                       | >220                   |
| 18                     | Senescent                | 96                       | >220                   |
| 19                     | Senescent                | 99                       | 86                     |
| 20                     | Cell death/immortalized  | n.d.<sup>e</sup>          | >220                   |
| 21                     | Senescent                | 96                       | 95                     |
| 22                     | Senescent                | n.d.<sup>e</sup>          | 86                     |
| 23                     | Senescent                | 99                       | 94                     |
| 24                     | Senescent                | 96                       | 101                    |
| 25                     | Cell death               | n.d.<sup>e</sup>          | 85                     |
| 26                     | Senescent                | 70                       | 92                     |
| 27                     | Senescent                | 86                       | 90                     |
| 28                     | Senescent                | 98                       | 100                    |
| 29                     | Senescent                | 69                       | 92                     |
| 30                     | Senescent                | 99                       | 102                    |
| 31                     | Senescent                | 96                       | >220                   |
| 32                     | Senescent                | 96                       | >220                   |
| 33                     | Cell death/immortalized  | n.d.<sup>e</sup>          | >220                   |
| 34                     | Senescent                | 96                       | 111                    |
| 35                     | Cell death/immortalized  | n.d.<sup>e</sup>          | >220                   |
| 36                     | Cell death/immortalized  | n.d.<sup>e</sup>          | >220                   |
| 37                     | Senescent                | n.d.<sup>e</sup>          | 88                     |
| 38                     | Senescent                | n.d.<sup>e</sup>          | 87                     |
| 39                     | Cell death               | n.d.<sup>e</sup>          | 86                     |
| 40                     | Senescent                | n.d.<sup>e</sup>          | 87                     |
| 41                     | Cell death/immortalized  | n.d.<sup>e</sup>          | >220                   |
| 42                     | Senescent/immortalized   | n.d.<sup>e</sup>          | >220                   |
| 43                     | Senescent                | 86                       | 92                     |
| 44                     | Senescent                | 92                       | 102                    |
| 45                     | Senescent                | 140                      |                       |
| 46                     | Cell death/immortalized  | n.d.<sup>e</sup>          | >220                   |

<sup>a</sup> Clone number indicates the number designated to MRC5hTERT clones with an extended lifespan.

<sup>b</sup> All extended-lifespan clones underwent crisis, during which cells either adopted a senescent morphology or underwent cell death. Cells that escaped crisis and were immortalized are indicated.

<sup>c</sup> X-gal activity was assessed at the time of growth arrest in representative clones that underwent senescent-like arrest at crisis.

<sup>d</sup> PD indicates the number of PDs at which expansion of the culture ceased. Immortal clones continued to proliferate beyond 220 PDs.

<sup>e</sup> n.d., not determined.

forced the permanent arrest of these clones. Overall our results suggest that up-regulation of p16<sup>INK4a</sup> and/or p21<sup>cip1</sup> slowed growth of MRC5hTERT cells at crisis, while repression of p16<sup>INK4a</sup> may have enabled immortalization.

**Expression and Phosphorylation of pRB during Immortalization of MRC5hTERT Cells**—The phosphorylation status of pRB was also investigated (Fig. 4a). Our results confirmed that pRB was in a hypophosphorylated state in senescent, non-transduced MRC5s (PD 63) (lane 7) and up-regulated and partially phosphorylated in earlier passage MRC5s (PD 57) (lane 6). In the MRC5hTERT-1 mass culture, pRB was down-regulated and in its active, hypophosphorylated form during crisis (lanes 2 and 3), then up-regulated and phosphorylated in post-crisis cells (lanes 4 and 5). These changes were consistent with the up-regulation of p16<sup>INK4a</sup> and p21<sup>cip1</sup> at crisis and down-regulation of these CDK inhibitors in post-crisis cells.

In addition to revealing phosphorylation status, the results in Fig. 4a suggested that the level of pRB expression was higher in post-crisis than pre-crisis MRC5hTERT cells. Further analysis of pRB expression in clones 12, 49 D1 and D2, as well as mass-cultured MRC5hTERT cells confirmed that pRB was indeed up-regulated in immortal MRC5hTERT cells compared with pre-crisis cells (Fig. 4b and additional data not shown). Elevated pRB was not merely a reflection of proliferative rate, as the level of expression did not correlate with doubling time at harvest (Fig. 4c). However, regression analysis confirmed an association between the level of pRB expression and the number of PDs at the time the assay was performed (p < 0.05). Hence pRB was up-regulated in parallel to down-regulation of p16<sup>INK4a</sup> during immortalization.

**MRC5hTERT Response to Genotoxic Stress**—Dysfunction of G1/S checkpoint control may render cells less sensitive to genotoxic stress, such as ionizing irradiation. Hence, expression of p21<sup>cip1</sup> and its transcriptional activator, p53, were investigated following exposure of MRC5hTERT cells to ionizing irradiation. In preliminary experiments, it was determined that p53 accumulated 3 h after exposure to 10 Gy irradiation, while p21<sup>cip1</sup> was maximally induced 24 h after irradiation in non-transduced MRC5 cells (data not shown). We next tested the response of pre- and post-crisis MRC5hTERT-1 mass-cultured cells, as well clones 2, 12, 49, and D1 to irradiation (Fig. 5a and additional data not shown). In all samples, including the immortal clone D1, p53 accumulated 3 h after exposure to 10 Gy irradiation, while p21<sup>cip1</sup> was induced 24 h after irradiation. These data indicate that the p53/p21<sup>cip1</sup> response to genotoxic stress was functionally normal in post-crisis MRC5hTERT cells, and that inactivation of this checkpoint was not necessary for immortalization. Phosphorylation and expression of pRB in response to
**Fig. 2.** Telomere length and telomerase enzyme activity in MRC5hTERT clones. a, TRAP assay for telomerase activity. Samples and PDs are indicated above the gels. IC, internal control used for quantitations. b, graphic representation of telomerase activity calculated from triplicate gels. Quantitations were performed as described under “Experimental Procedures.” c, TRF analysis of MRC5hTERT clones. Pre- (≤ 92 PD) and post-crisis (≥ 141 PD) time points are shown for clones that underwent a period of cell death prior to immortalization. Mean TRF for each sample, calculated as described under “Experimental Procedures,” is indicated below the gel. Molecular weights determined from a DNA size marker are indicated in Kbp to the left of the gel.
irradiation was also investigated. Western blot analysis demonstrated that pRB was phosphorylated prior to irradiation, then became hypophosphorylated 18 h after irradiation. This phosphorylation pattern was observed in non-transduced MRC5 cells, as well as pre- and post-crisis MRC5hTERT cells, indicating that phosphorylation of pRB in response to genotoxic stress was unperturbed during immortalization (Fig. 5b).

**MRC5hTERT Response to Oncogenic ras**—Previous studies have shown that p16INK4a may be induced by overexpression of oncogenic ras in primary human fibroblasts (12). Hence, to determine whether repression of p16INK4a in immortal cells was reversible, MRC5hTERT-1 cells were transduced with a retroviral vector encoding oncogenic N-ras plus a YFP. As a control, MRC5hTERT-1 cells were transduced with a vector encoding YFP alone. MRC5hTERTs that had just escaped crisis (135 PD) (MRC5hTERT (135)) and expressed low levels of p16INK4a, as well as immortal MRC5hTERTs at 414 PD (MRC5hTERT (414)), which were p16INK4a-negative, were used in these investigations (Fig. 6). FACS analysis for expression of YFP demonstrated that at least 90% of the MRC5hTERT-1 cells were transduced with the YFP and N-ras-YFP vectors (Fig. 6a). Western blot analysis, performed 4 days after retroviral transduction revealed that p16INK4a was up-regulated in N-ras-transduced MRC5hTERT (135), but not MRC5hTERT (414) cells (Fig. 6b). No consistent change in expression of p21\(^{cip1}\) was detected in either cell population following transduction with the N-ras-YFP vector (data not shown). Induction of p16INK4a in MRC5hTERT (135) cells was associated with a growth arrest within 11 days of transduction (Fig. 6c). The arrested cells adopted a senescent-like morphology and expressed SA-\(\beta\)gal (Fig. 6d). In contrast to the earlier passage cells, late passage MRC5hTERT (414) cells transduced with N-ras-YFP (MRC5hTERT(414)(N-ras)) did not undergo arrest and did not exhibit a statistically significant increase in SA-\(\beta\)gal activity.

MRC5hTERT (414) and MRC5hTERT (135) cells, transduced with the N-ras-YFP and YFP vectors were also tested for evidence of transformation. When plated in soft agarose, MRC5hTERT(414)YFP, but not MRC5hTERT(135)YFP cells formed clusters and small colonies, which measured up to 200 \(\mu\)m in diameter. However, the cloning efficiency (0.05%) was much lower than other transformed cell lines we have tested (29). The colony forming efficiency of MRC5hTERT(414)Nras cells was significantly higher (4-fold) than MRC5hTERT(414)YFP cells (\(p = 0.0017\)) (Fig. 6e). MRC5hTERT(414)Nras colonies were also larger (up to 500 \(\mu\)m in diameter) than MRC5hTERT(414)YFP colonies. Neither MRC5hTERT(414)YFP nor MRC5hTERT(414)-Nras cells formed tumors when 5 \(\times\) 10\(^6\) cell were subcutaneously implanted in nude mice. The mice were monitored for tumor formation for 5 months. In contrast, tumors formed within 3 weeks in control mice that were inoculated with 5 \(\times\) 10\(^6\) HT1080 fibrosarcoma cells. Thus, the combination of telomerase activity, inactivation of p16INK4a, and expression of oncogenic N-ras in MRC5 cells was not sufficient for tumor formation.

**MRC5hTERT Response to p16INK4a Expression**—To determine whether loss of p16INK4a was instrumental in the immortalization process, immortal and pre-crisis MRC5hTERT cells were transduced with retroviral vectors encoding p16wt and p16M53T (43). Transduction with the p16wt vector was sufficient to halt proliferation of immortal MRC5hTERT-1 (MRC5hTERT (400)) cells. Indeed, the p16wt vector was as effective in immortal MRC5hTERT cells as pre-crisis cells (MRC5hTERT (75)) cells (Fig. 7e) and normal MRC5s (additional data not shown). Growth-arrested cells adopted a senescent morphology and exhibited SA-\(\beta\)gal activity (Fig. 7b). The Met to Thr mutation in the p16M53T vector completely abrogated the growth suppressive activity of p16INK4a, as previously reported (43). These results demonstrate that reconstitution of p16INK4a restores immortal MRC5hTERT cells to a phenotype that is indistinguishable from pre-crisis cells and support the possibility that loss of p16INK4a was a critical event in the immortalization process.

**DISCUSSION**

These investigations confirmed that MRC5 fibroblasts were not universally immortalized by ectopic expression of telomerase. Furthermore, clonal analyses revealed that only 28% of MRC5hTERT cells that bypassed senescence were eventually immortalized, as the remainder of the clones succumbed to a telomere-independent crisis. This is consistent with the rate of immortalization determined in our previous study of 26 MRC5hTERT clones (45). A 28% rate of escape from crisis is very high compared with the rate that SV40 T-transformed fibroblasts escaped crisis; estimated to be 1/10\(^5\) (46). Our result is consistent with the possibility that the mechanism(s) that enabled MRC5hTERT cells to escape crisis were not as stringently regulated as de-repression of telomerase or ALT, which enables virally transformed cells to escape crisis (38–40, 47).
Two cellular fates were observed at crisis; clones succumbed to either a senescent-like growth arrest, or cell death. Both of these phenotypes were previously observed in crisis cultures of virally transformed cells (36, 37). The onset and duration of crisis was variable among the clones. Considering the high rate of escape from crisis, it is conceivable that clonal variations could potentially mask crisis in mass cultures. Indeed, this may explain the spectrum of growth patterns observed among 4

**FIG. 4. Phosphorylation and expression of pRB.** a, Western blot analysis of pRB in MRC5 and MRC5hTERT-1 mass-cultured cells at different time points. The change of phosphorylation status was resolved by electrophoresis through a 7% polyacrylamide gel. b, Western analysis of total pRB and actin (loading control) expression in MRC5hTERT-1 mass culture and clones, resolved by electrophoresis through a 15% polyacrylamide gel. c, Regression analysis was performed using data obtained by densitometric analysis of the Western blot shown in b.Doubling time was calculated by taking the average number of PD/day from a least three time points around the time the cells were harvested. pRB expression was normalized to actin and analyzed using GraphPad Prism v4.0.

**FIG. 5. p53, p21cip1, and pRB response to irradiation.** Cells were irradiated at 10 Gy while in log phase growth, then returned to culture and harvested at 3, 18, and 24 h for analysis of p53, pRB, and p21cip1, respectively. a, Western blot analysis of p21cip1 and p53. Total actin expression was used as a loading control. b, Western blot analysis of pRB expression and phosphorylation. Ponceau S staining is shown as a loading control.
MRC5hTERT mass cultures in our previous study (32), as well as some apparent discrepancies in the literature concerning the ability of hTERT to immortalize fibroblasts. Analysis of telomerase activity in MRC5hTERT clones indicates that crisis was unlikely due to the level of telomerase enzyme activity. A suboptimal level of telomerase was detected in MRC5hTERT clone 1, which underwent a senescent-like arrest at crisis. However, this clone was an exception, as it expressed a lower level of telomerase than all other clones tested and was the only clone that did not have extended telomeres. In contrast to clone 1, another 2 clones that underwent delayed senescence at crisis expressed higher levels of telomerase and had hyperextended telomeres (greater than 20 Kbp). A recent report has shown that very high levels of exogenously expressed telomerase may induce a senescent-like arrest in hTERT-transduced fibroblasts (48). However, high lev-

**Fig. 6. N-ras transduction of MRC5hTERT-1 cells.** MRC5hTERT cells at 135 or 414 PD were transduced with N-ras-YFP and YFP retroviral vectors. A. FACS analysis for YFP expression, performed four days after retroviral transduction. B. Western blot analysis for p16INK4a expression performed four days after transduction with N-ras-YFP and YFP retroviral vectors. Expression of actin is shown as a loading control. C. Proliferation of transduced MRC5hTERT (135) and MRC5hTERT (414) cells. The 3-day period for retroviral infection is indicated by the bar below the graph. D. MRC5hTERT (135) and MRC5hTERT (414) cells were assayed for SA-β-gal activity 11 days after retroviral transduction with N-ras-YFP and YFP retroviral vectors. Cells with SA-β-gal activity stained blue (x-gal positive) and were scored under an inverted microscope as a percentage of the total cell population. Values shown are averages, with S.D., calculated from at least three fields of 100–200 cells per cell line. **, Student’s t test indicated MRC5hTERT(135)YFP and MRC5hTERT(135)Nras to be statistically different (p = 0.0002). E. Cells were plated in soft agarose at 10,000/ml in three independent experiments. Triplicate plates were set up in each experiment. Colonies were scored under an inverted microscope. Values indicate the average and S.D. calculated from the three experiments. No colonies were formed by MRC5hTERT(135)YFP and MRC5hTERT(135)Nras cells. **, Student’s t test indicated the number of colonies generated by MRC5hTERT(135)YFP and MRC5hTERT(135)Nras to be statistically different (p = 0.0017).
were scored by counting 50–2 translating the mean from three fields. Data represent means and S.D. from
activity 9
our report and the study by Gorbunova
Indeed the highest levels of telomerase were detected in some
els of telomerase did not appear to be the cause of senescent-
lke arrest or crisis in MRC5hTERT cultures, since there was
result may reflect inherent differences in MRC5 lung fibro-
also as in oncogene-induced premature senescence (2, 3, 12, 13, 49). Up-regu-
ulation of p21\(^{ci/p1}\) has also been associated with growth arrest induced by oxidative stress (50), while p16\(^{INK4a}\) may be induced by suboptimal culture conditions, such as growth in chemically defined media with low (0.25%) serum (30). Suboptimal culture conditions were unlikely to be the cause of crisis in MRC5hTERT cultures, since 10% serum was included in the culture media and the onset of crisis in MRC5hTERT cells and senescence in MRC5 cells was very similar in 2 different media formulations. In addition, non-transduced MRC5s underwent replicative senescence at ~65 PDL when telomeres were around 5.0 Kbp, which is consistent with expectations for fibroblasts grown under optimal culture conditions.

Following crisis, p16\(^{INK4a}\) and p21\(^{ci/p1}\) were down regulated, while pRB became phosphorylated. Our result showing repression of p16\(^{INK4a}\) in post-crisis MRC5hTERT cells is consistent with a recent report that demonstrated down-regulation of p16\(^{INK4a}\) in post-crisis human foreskin fibroblasts that were immortalized with hTERT (35). Our results showing that restoration of p16\(^{INK4a}\) in post-crisis MRC5hTERT cells induced growth arrest and features of senescence, provides further evidence that loss of p16\(^{INK4a}\) was a critical event in the immortalization process. The functional significance of the down-regulation of p21\(^{ci/p1}\) in MRC5hTERT cells is less clear, since p21\(^{ci/p1}\) was repressed in clones that escaped crisis, as well as in clones that permanently arrested in a senescent-like state. Furthermore, p21\(^{ci/p1}\) was readily induced by irradiation in very late passage cells. In the study by Noble et al. (35) a defective p53/p21\(^{ci/p1}\) response to DNA damage was found in 1 of 3 hTERT-immortalized clones. In contrast, we found no defects in the p53/p21\(^{ci/p1}\) DNA damage response in the post-crisis MRC5hTERT mass culture and four post-crisis clones, including one clone that was passaged for more than 400 PDs. This result may reflect inherent differences in MRC5 lung fibroblasts compared with the foreskin fibroblasts employed in the study by Noble et al. (35).

It is noteworthy that the pattern of p16\(^{INK4a}\) and p21\(^{ci/p1}\) expression observed in MRC5hTERT clones that underwent senescent-like arrest at crisis was analogous to normal fibroblasts during replicative senescence, where p21\(^{ci/p1}\) is transiently induced and high levels of p16\(^{INK4a}\) are sustained (51). One proposed explanation for these observations is that transient high levels of p21\(^{ci/p1}\) act to initiate senescence, while high levels of p16\(^{INK4a}\) sustain the senescent phenotype (51). In contrast to p21\(^{ci/p1}\), inactivation of p16\(^{INK4a}\) corresponded well with immortalization. Expression of p16\(^{INK4a}\) was reduced in clones that escaped crisis, but not clones that arrested in a senescent-like state. However, the timing of p16\(^{INK4a}\) inactivation did not precisely correspond with escape from crisis, as a low level of p16\(^{INK4a}\) was detected in early post-crisis cells. This may be a reflection of the long half-life of p16\(^{INK4a}\) protein, unsynchronized repression of p16\(^{INK4a}\) in subsets of cells and/or retention of p16\(^{INK4a}\) in subpopulations of cells that did not escape crisis and were carried over in the culture. These possibilities are consistent with the gradual outgrowth of p16\(^{INK4a}\)-negative cells in the mass culture.

It was previously shown that expression of hTERT does not overcome ras-induced premature senescence in human fibroblasts (52). The present study demonstrates that immortal p16\(^{INK4a}\)-negative MRC5hTERT cells were refractory to ras-induced de-repression of p16\(^{INK4a}\) and senescent-like growth.
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arrest. Our results are in agreement with recent studies that showed fibroblasts derived from individuals who were genetically deficient at the INK4a locus were resistant to ras-induced growth arrest (43, 53). Similar to immortal MRC5hTERT cells, the cells from the INK4a-deficient individuals retained p53/p21^{kip1} function. These results strongly implicate p16^{INK4a} as the critical mediator of ras-induced growth arrest. However, a contrasting report that examined the effects of p16^{INK4a} inactivation by overexpressing a mutant cdk4-cyclin D fusion protein concluded that inactivation of p21^{kip1} in addition to abrogation of p16^{INK4a} was necessary for overcoming ras induced growth arrest (54). There are several possible explanations for this apparent discrepancy, such as different levels of ras expression, different methodologies and vectors, and/or spontaneous molecular changes or inherent differences in the various cell strains employed.

Transduction with oncogenic ras facilitated anchorage independent growth of p16^{INK4a}-negative MRC5hTERT cells. However, the cloning efficiency was very low and the colonies were very small compared with other transformed cell lines that we have tested (29). Brookes et al. (53), reported more robust colony formation when INK4a-deficient fibroblasts were co-transfected with hTERT and ras, while Wei et al. (54) indicated that ablation of both p16^{INK4a} and p21^{kip1} were necessary for anchorage-independent growth of hTERT-transduced fibroblasts. Considerable variations in the materials and methods used for this assay are likely to account for differences obtained in these independent investigations. Despite the variations among these reports on the requirements for anchorage independent growth, there is a long-standing consensus that several molecular alterations are required for acquisition of a tumorigenic phenotype (1). Our results, showing that N-ras transduced p16^{INK4a}-negative MRC5hTERT cells were non-tumorigenic in immunocompromised mice are consistent with this notion. It is likely that inactivation of the P53/p21 pathway complicated by our results and others, which have demonstrated selection for p16^{INK4a} inactivation during immortalization of human fibroblasts (33, 35, 57, 58). Specifically, our investigations indicate that inactivation of p16^{INK4a} may be necessary to overcome telomere-independent crisis, which appears to be an integral event during hTERT-immortalized MRC5 fibroblasts.

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