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Journal

Cell Cycle, 6(15)

ISSN

1538-4101

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Publication Date

2007-08-01

DOI

10.4161/cc.6.15.4519

Peer reviewed

Cell Cycle

ISSN: 1538-4101 (Print) 1551-4005 (Online) Journal homepage: https://www.tandfonline.com/loi/kccy20

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To cite this article: James C. Garbe, Charles R. Holst, Ekaterina Bassett, Thea D. Tlsty & Martha R. Stampfer (2007) Inactivation of p53 Function in Cultured Human Mammary Epithelial Cells Turns the Telomere-Length Dependent Senescence Barrier from Agonescence into Crisis, Cell Cycle, 6:15, 1927-1936, DOI: 10.4161/cc.6.15.4519

To link to this article: https://doi.org/10.4161/cc.6.15.4519

[Cell Cycle 6:15, 1927-1936, 1 August 2007]; ©2007 Landes Bioscience

Report

Inactivation of p53 Function in Cultured Human Mammary Epithelial Cells Turns the Telomere-Length Dependent Senescence Barrier From Agonescence into Crisis

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Original manuscript submitted: 05/25/07
Manuscript accepted: 05/30/07

Previously published online as a *Cell Cycle* E-publication:
http://www.landesbioscience.com/journals/cc/abstract.php?id=4519

KEY WORDS

p53, agonescence, crisis, senescence, genomic instability, stasis

ABBREVIATIONS

CKI	cyclin dependent kinase inhibitor
GSE	genetic suppressor element
HMEC	human mammary epithelial cells
LI	labeling index
OIS	oncogene-induced senescence
PD	population doublings
Rb	retinoblastoma protein
SA-β-Gal	senescence associated β-galactosidase
TRF	terminal restriction fragment

ACKNOWLEDGEMENTS

See page 1934.

ABSTRACT

Cultured human mammary epithelial cells (HMEC) encounter two distinct barriers to indefinite growth. The first barrier, originally termed selection, can be overcome through loss of expression of the cyclin-dependent kinase inhibitor $p16^{INK4A}$. The resultant $p16^{-}$, $p53^{+}$ post-selection HMEC encounter a second barrier, termed agonescence, associated with critically shortened telomeres and widespread chromosomal aberrations. Although some cell death is present at agonescence, the majority of the population retains long-term viability. We now show that abrogation of p53 function in post-selection HMEC inactivates cell cycle checkpoints and changes the mostly viable agonescence barrier into a crisis-like barrier with massive cell death. In contrast, inactivation of p53 does not affect the ability of HMEC to overcome the first barrier. These data indicate that agonescence and crisis represent two different forms of a telomere-length dependent proliferation barrier. Altogether, our data suggest a modified model of HMEC senescence barriers. We propose that the first barrier is Rb-mediated and largely or completely independent of telomere length. This barrier is now being termed stasis, for stress-associated senescence. The second barrier (agonescence or crisis) results from ongoing telomere erosion leading to critically short telomeres and telomere dysfunction.

INTRODUCTION

Human cells cultured from normal somatic tissues express senescence, i.e., a limited proliferative potential; spontaneous immortal transformation is virtually unknown. The mechanisms responsible for enforcing this finite lifespan have not been clearly defined. Critically shortened telomeres resulting from telomerase repression, and responses to various stresses and/or DNA damage have been proposed as major limiting factors.[1-5] Additionally, oncogene-induced senescence (OIS) can be induced by exposure to certain oncogenes.[6-9]

A commonly used model of immortal transformation of cultured human cells posits overcoming at least two barriers, which have been called senescence or M1, and crisis or M2.[10,11] As originally proposed, the first barrier, senescence, is postulated to be due to shortened telomeres signaling activation of p53 and retinoblastoma (Rb) controlled cell cycle checkpoints, causing a viable arrest. Extended life cultures, usually obtained through exposure to viral oncogenes that functionally inactivate both Rb and p53, eventually reach the M2 barrier and undergo crisis, i.e., critically shortened telomeres producing genomic instability and cell death. Overcoming crisis has been thought to require acquiring a rare mutation during crisis that reactivates telomerase activity.[12] This model has been complicated by the demonstration of nontelomere length dependent senescence resulting from oncogenic and other stresses in many cultured human cell types.[13-17]

Our data using cultured human mammary epithelial cells (HMEC), along with data from other human cell systems, suggests a modified model of the senescence barriers encountered by finite human cells in vitro. HMEC derived from reduction mammoplasty tissue have undergone ~15–60 population doublings (PD) in vitro prior to encountering a first proliferation barrier, with the variation in the number of PD attained dependent upon culture conditions (Garbe J, Stampfer M, unpublished).[2,18,19] HMEC arrested at this barrier show a low labeling index (LI) of ~2%, viable arrest in G_1, normal karyotypes, a variable mean telomere restriction fragment (TRF) length of ~6–8 kb, expression of senescence-associated β-galactosidase activity (SA-β-Gal), and a large, flat, vacuolated morphology.[20,21] Under some culture conditions, proliferating cells have spontaneously emerged from arrested cell populations.[19,22] We originally called this proliferative barrier

selection, and the emergent proliferative population post-selection.[19] Post-selection cells lack expression of the cyclin-dependent kinase inhibitor (CKI) $p16^{INK4A}$, associated with methylation of the p16 promoter.[23] Post-selection HMEC encounter a second proliferation barrier after ~30–70 additional PD. This barrier, recently termed agonescence, is associated with critically shortened telomeres and widespread chromosomal abnormalities, including telomere associations.[21,24] Agonescent HMEC show a moderate LI of ~15%, a mostly viable arrest at all phases of the cell cycle along with some cell death, expression of SA-β-Gal, and a large, flat, vacuolated morphology. A situation characteristic of crisis, i.e., a high LI and massive cell death, was not observed in our finite lifespan HMEC populations. In this study we tested the hypothesis that the functional p53 present in post-selection HMEC induces a senescence response in the presence of critically short telomeres, thereby preventing the massive cell death and ongoing genomic instability associated with crisis.

We now demonstrate that the presence of functional p53 represents the distinction between agonescence and crisis. Abrogation of p53 function in post-selection HMEC inactivates cell cycle checkpoints and changes the mostly viable agonescence barrier into a crisis-like barrier with massive cell death. Abrogation of p53 function prior to the first barrier did not affect growth of the HMEC population. Altogether, our data suggest a modified model of HMEC senescence barriers using molecular defined nomenclature (see Fig. 6). In this model, the first barrier (originally termed selection) represents a Rb-mediated, nontelomere-length dependent, stress associated arrest, which we are calling stasis.[1] Phenotypic markers suggest that stasis is most similar to what has been called senescence or M1 in other cell systems. The second barrier is due to critically shortened telomeres producing telomere dysfunction. This barrier manifests as the recently described agonescence when p53 is functional, and as crisis in the absence of p53-dependent checkpoint arrest.

MATERIALS AND METHODS

Cell culture. Finite lifespan prestasis HMEC strain 184 (batch F) and post-selection HMEC strains 184 (batch B, agonescence at ~passage 15) and 48R (batch S, agonescence at ~passage 23) were obtained from reduction mammoplasty tissue that showed no epithelial cell pathology. Cells derived from primary tissues were grown in serum-containing MM medium, or serum-free MCDB 170 medium (MEGM, Clonetics Division of Cambrex, Walkersville, MD), as described.[18] Post-selection HMEC were cultured in MCDB 170 as described.[19,25,26] Labeling index was determined by addition of 3H-thymidine (0.5 μCi/ml) for 4 or 24 hr following refeeding, and visualization by autoradiography was as described.[27] Immunohistochemical analysis for p16 expression was performed as described using the JC8 antibody.[28] SA-β-Gal activity was determined as described.[29] In growing populations, each passage represents ~3–4 PD. Complete details on the derivation and culture of these HMEC can be found on our web site, www.lbl.gov/~mrgs/mindex.html.

Retroviral transduction. The pBABE-GSE22-puro plasmid, encoding a p53 genetic suppressor element (GSE) in a retroviral vector[30] was provided by Drs. Andrei Gudkov and Peter Chumakov, U. Ill., Chicago. GSE22 encodes the p53 nucleotides 937–1199, and the resultant peptide acts as a dominant negative suppressor by inhibiting the p53 tetramerization domain. Retroviral stocks were generated by transient cotransfection of the vector plasmid along with a plasmid encoding packaging functions into the 293 cell line.[31] Retroviral supernatants were collected in serum free MCDB 170 media containing 0.1% bovine serum albumin, filter sterilized and stored at -80°C. For lentivirus infections, the GSE22 insert was cloned into the pRRL.SIN-18 vector[32] and virus stocks produced as described.[33] Viral infection of 184 and 48R HMEC cultures was in MCDB 170 media containing 0.1% bovine serum albumin and 2.0 μg/ml polybrene (Sigma).

p53 function. For G_1 checkpoint assays, HMEC in log phase growth were exposed to 10 Gy of ionizing radiation from a Pantak II x-ray generator at 150 kV and 20 mA with beam filtration of 1.02 mm aluminum and 0.5 mm copper. Dosimetry was performed using a NIST-calibrated Victoreen condenser R-meter. Mock irradiated and irradiated cells were collected at 24 and 48 hrs post treatment and prepared for FACS analysis. For a spindle assembly checkpoint assays, HMEC in log phase growth were cultured in media containing 50 ng/ml colcemid (Karyomax, Life Technologies, Bethesda, MD). Treated cultures were refed every 24 hrs and samples were collected and prepared for FACS analysis at 0, 24, 48, 72, and 96 hrs. All cells were labeled with 10 μM BrdU (Sigma, St. Louis, MO) for 4 hours immediately prior to harvest. Analyses of BrdU incorporation and total DNA content were performed using a Becton-Dickinson flow cytometer. All analyzed events were gated to remove debris and aggregates. The fractions of BrdU(+) cells with specific DNA contents were determined by dividing the number of BrdU(+) events by the total number of gated events.

DNA damage assays. Subconfluent HMEC grown on 4-well chamber slides were either irradiated with 10 Gy of ionizing radiation, or mock-irradiated, and allowed to recover at 37°C for 6 h. The cells were then fixed with 4% paraformaldehyde, and permeabilized with 0.1% Triton X-100 (Sigma). The slides were blocked with 10% goat serum (Sigma) in CAS-Block (Zymed), and incubated with primary antibodies, against the serine 15 phosphorylated form of p53 (#9284, Cell Signaling Technology, Danvers, MA), the serine 139 phosphorylated form of H2AX (Clone JBW301, Chemicon, Temecula, CA), and 53BP1 (#A300-273A, Bethyl Laboratories, Montgomery, TX), or normal mouse or rabbit IgG (Invitrogen) as negative controls. After extensive washing with 0.1% Tween 20 (Sigma) in PBS, the slides were incubated with Alexa 488-conjugated anti-mouse IgG, or Alexa 594-conjugated anti-rabbit IgG (Invitrogen). Stained cells were visualized using a Zeiss Axiovert 200M inverted fluorescence microscope and imaged by a Retiga EX camera (Q-Imaging) and Image-Pro® Plus software (MediaCybernetics).

Telomerase and mean TRF length assays. Telomerase assays were performed as described[20] using the TRAP-EZE telomerase detection kit (Chemicon) and 2 μg of protein per assay. The telomerase products were visualized by Syber Green staining (Molecular Probes, Eugene, OR) and detected using a STORM imaging system (Molecular Dynamics, Sunnyvale, CA). DNA isolation and mean TRF analysis were performed as previously described using 3 μg of digested genomic DNA.[34,35]

RESULTS

p53 function is inactivated following transduction of GSE22. To test the hypothesis that functional p53 prevented crisis-associated massive cell death, we inactivated p53 function in post-selection HMEC using the p53 dominant negative genetic suppressor element GSE22.[30] Post-selection 184B HMEC were transduced with retroviral vectors containing GSE22 or empty control vector at passage

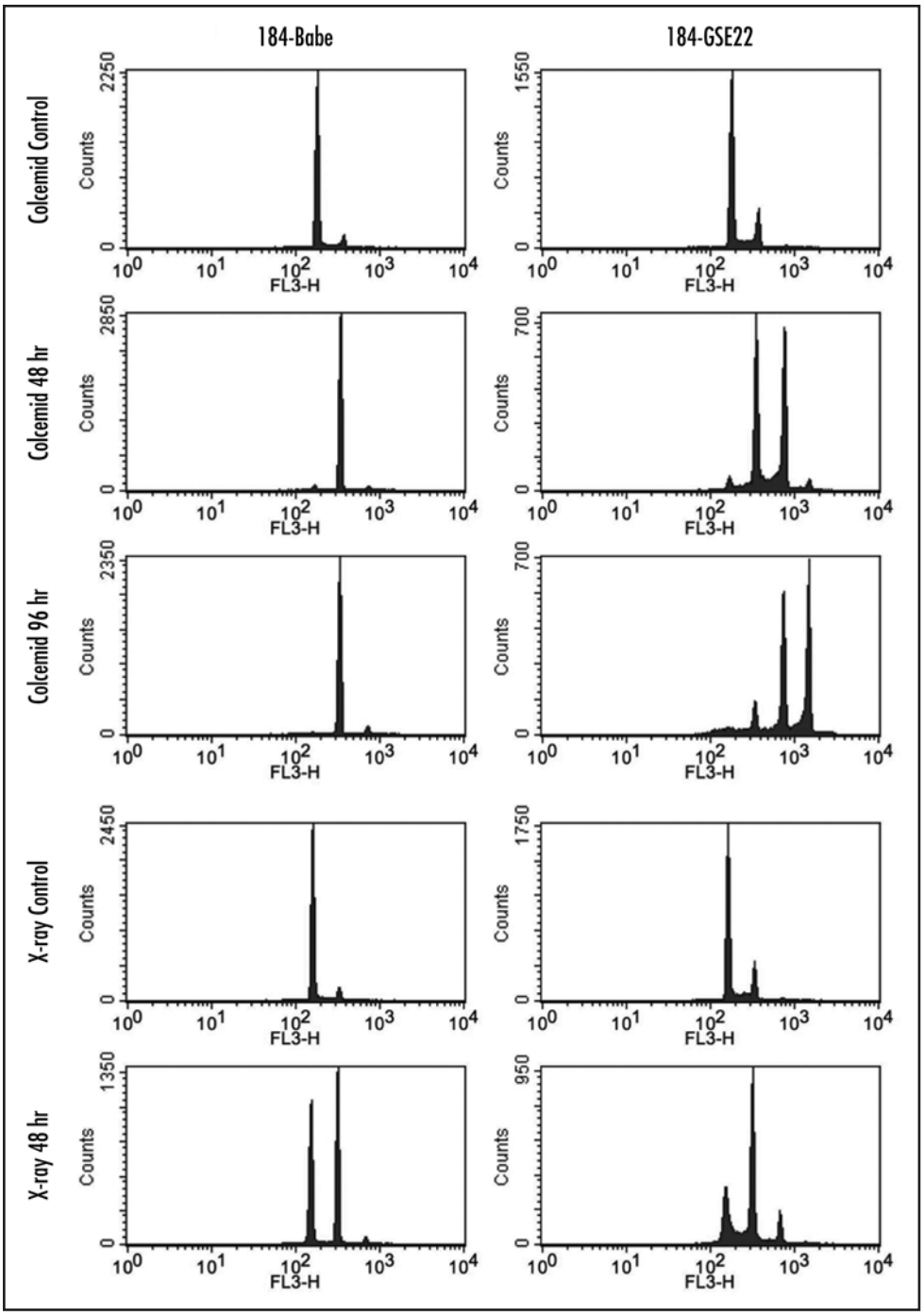

Figure 1. Transduction with GSE22 abrogates p53 function in post-selection 184 HMEC. 184B HMEC infected with GSE22-containing or control (Babe) vectors at passage 5 were analyzed by FACS analysis at passage 9 for DNA content in response to (A) 50 ng/ml colcemid and (B) 10 Gy of ionizing radiation.

5; ost-selection 48RS HMEC were transduced at passage 11. After selection with puromycin, the GSE transduced and control cells were assayed for p53 function following exposure to ionizing radiation or colcemid. Cells exposed to 10 Gy of x-irradiation were examined by FACS analysis after 24 and 48 hours, and cells exposed to 50 ng/ml colcemid were examined after 24, 48, 72 and 96 hrs. As shown in Figure 1 and Table 1, in unexposed cycling populations at passage 9, 184B-GSE22 compared to control 184B-Babe showed a modest increase in cells in S phase (~30% vs. 19%) and an increased fraction with a ≥4N DNA content (~3.5% vs. 0.6%). Following irradiation, 184B-Babe showed growth arrest in both G_1 and G_2, with few cells in S phase or with >4N DNA. In contrast, 184B-GSE22 failed to exhibit arrest; populations displayed ongoing DNA synthesis, with a major DNA peak at 4N as well as some cells with 8N DNA content. Following exposure to colcemid, the control population showed nearly complete growth arrest with 4N DNA content by 96 hr, with a small peak at 8N DNA content and almost no BrdU incorporation. In contrast, the 184B-GSE22 population continued to initiate DNA synthesis in the absence of mitosis and accumulated cells with ≥8N DNA content. Similar results were seen with specimen 48RS (data not shown).

These data indicate that p53 checkpoint-arresting functions have been abrogated in the GSE22-transduced populations. Additionally, the abundant p53 protein previously shown to be present in these post-selection HMEC,[35,36] does not show significant checkpoint-arresting activity in the absence of activating stimuli such as irradiation or colcemid.

p53 inactivation affects growth and morphology of post-selection HMEC. To determine the effect of abrogation of p53 function on growth capacity before and at agonescence, GSE22-transduced and control 184B HMEC populations were assayed for percentage of cells synthesizing DNA during 4 hr and 24 hr time periods starting from passage 8. Cells were also observed for morphology and viability.

There were no initial obvious differences between the GSE22-transduced and control cells at early passages after transduction. Both cell populations retained the typical cobblestone morphology of epithelial cells (Fig. 2A and C) and showed the same 24 hr LI of 93% (Table 2). The 184B-GSE22 population displayed a slight initial increased growth rate compared to 184B-Babe, which became more pronounced with ongoing subculture (Fig. 2G). By passage 12, there was a significant difference in the 24 hr LI between 184B-Babe (40%) vs. 184B-GSE22 (76%) (Table 2). As the control 184B-Babe population approached agonescence, its LI continued to decrease and its morphology changed, with an increasing percentage of the population exhibiting a senescent morphology of large, flat, vacuolated cells (Table 2 and Fig. 2B). By passage 15 there was no net increase in cell number and the 24 hr LI was ~15%. In contrast, 184B-GSE22 populations retained their small cobblestone morphology and a higher LI for an additional 2–3

Table 1 **BrdU incorporation of 184B-Babe and 184B-GSE22 after exposure to irradiation or colcemid at passage 9**

Cell	Control		Colcemid 96 hr		Control		X-ray 48 hr	
	% total	% BrdU+	% total	% BrdU+	% total	% BrdU+	% total	% BrdU+
184B-Babe								
<2n	0.48	0.03	0.32	0.02	0.51	0.05	0.32	0.00
2n	81.66	6.33	1.82	0.07	82.70	5.05	47.70	1.52
2n>4n	9.41	9.28	2.85	0.06	8.06	7.87	3.09	2.33
4n	7.84	4.38	88.59	0.19	8.17	4.67	44.33	4.02
>4n	0.61	0.34	6.41	0.95	0.56	0.35	4.55	2.58
Total	100	20.36	100	1.29	100	17.98	100	10.45
184B-GSE22								
<2n	0.38	0.08	2.88	0.32	0.44	0.03	1.27	0.20
2n	63.99	7.27	3.41	0.32	64.22	6.54	27.13	7.65
2n>4n	13.46	12.80	4.35	0.44	14.03	13.27	15.03	12.92
4n	19.02	8.91	7.95	0.49	17.45	7.26	41.13	9.01
>4n	3.16	2.04	81.40	26.19	3.86	2.22	15.44	8.63
Total	100	31.11	100	27.76	100	29.31	100	38.42

passages. By passage 15, the cell population contained a mixture of small proliferating cells along with large, vacuolated cells (Fig. 2D). The LI, 44%, was similar to that reported for cells in crisis.[37] With continuing time, either following subculture (data not shown), or observing the cell population remaining at passage 15, large vacuolated cells became predominant, with cultures eventually showing abundant cell debris (Fig. 2E and F) and a slowly declining LI (Table 2). These morphological changes are similar to those reported for cells in crisis. Both 184B-Babe and 184B-GSE22 showed an increasing percentage of SA-β-Gal(+) cells with passage. By passage 15, virtually all control cells were SA-β-Gal(+) (data not shown) as were the 184B-GSE22 cells with a senescent morphology (Fig. 2H).

To further demonstrate that loss of p53 function was responsible for the high LI seen in the late passage 184B-GSE22 cultures, GSE22 was transduced into an already agonescent culture of 184B HMEC at passage 15 using a lentiviral vector,[38] which allows infection of both dividing and nondividing cells. Seventy-two hours after infection the GSE22 transduced cultures had a 24 hr LI of 67%. In contrast, the cells transduced with the control lentivirus alone had a 24 hr LI of only 8%. Thus inactivation of p53 even at agonescence will allow growth-arrested cells exhibiting telomere dysfunction to resume DNA synthesis.

These data indicate that the abrogation of p53 function in post-selection HMEC initially does not have a significant effect on growth rate, but with continued proliferation leading to telomere erosion, eliminates the growth-restraining consequences of p53 activation, turning the largely viable agonescence arrest into a situation of crisis; i.e., high LI leading to massive cell death. We have not observed any instances of immortal clones arising from the 184B-GSE22 populations at crisis, based on observing the fate of more than 2×10^8 cells brought to crisis and maintained in culture for six months.

Post-selection HMEC at agonescence show evidence of a DNA damage response. To support the hypothesis that telomere dysfunction at agonescence is eliciting a DNA damage response that activates p53, we examined young and agonescent post-selection HMEC for γH2AX and 53BP1, markers associated with DNA damage,[39] and for activated p53 (phosphorylated on serine 15). Figure 3 shows the results for 184B and 48RS. In both cases, the agonescent culture was one passage away from no net increase in cell number, whereas the young cultures were 8 and 14 passages away respectively. As expected, numerous colocalized foci of 53BP1 and γH2AX were seen in the cells at agonescence and after x-irradiation, and expression of activated p53 was detected. In the young cultures, ~90% of the 48RS cells had 0–1 focus/nucleus, and faint expression of activated p53, while the 184B cultures had ~40% with 0–1 focus/nucleus, with no detectable expression of activated p53. Possibly, the greater expression of DNA damage foci in the young 184B cultures may reflect their closer proximity to agonescence. These data indicate that HMEC at agonescence show evidence of a DNA damage response and activation of p53.

Telomerase activity and mean TRF length. 184B-GSE22 and 184B-Babe populations were assayed for telomerase activity following retroviral infection at different passages. No activity was detected in control populations at any passage level. In two separate experiments, faint or no telomerase activity was seen in 184B-GSE22 (Fig. 4A). While faint activity could be detected at passage 7, this was largely absent at the passages closer to crisis (passages 13–14). Thus, inactivation of p53 function was insufficient to produce sustained reactivation of telomerase activity in $p16^-$ post-selection HMEC, consistent with the observed lack of immortal transformation. However, it is possible that inactivation of p53 function may elicit a transient increase in telomerase activity. Analysis of mean TRF length showed a reduced telomere length in 184B-GSE22 compared to 184B-Babe (3.1 vs. 3.8 kb), as well as a fainter signal, consistent with the extended proliferation of the p53-inactivated population (Fig. 4B).

p53 inactivation does not affect growth of prestasis HMEC. In other human cell types, e.g., keratinocytes and astrocytes, inactivation of p53, as well as p16 function, was necessary to overcome a telomere length independent proliferative barrier and permit efficient immortalization by hTERT transduction.[13,40] We have previously shown that $p16^-$ post-selection HMEC, which retain p53 function, could

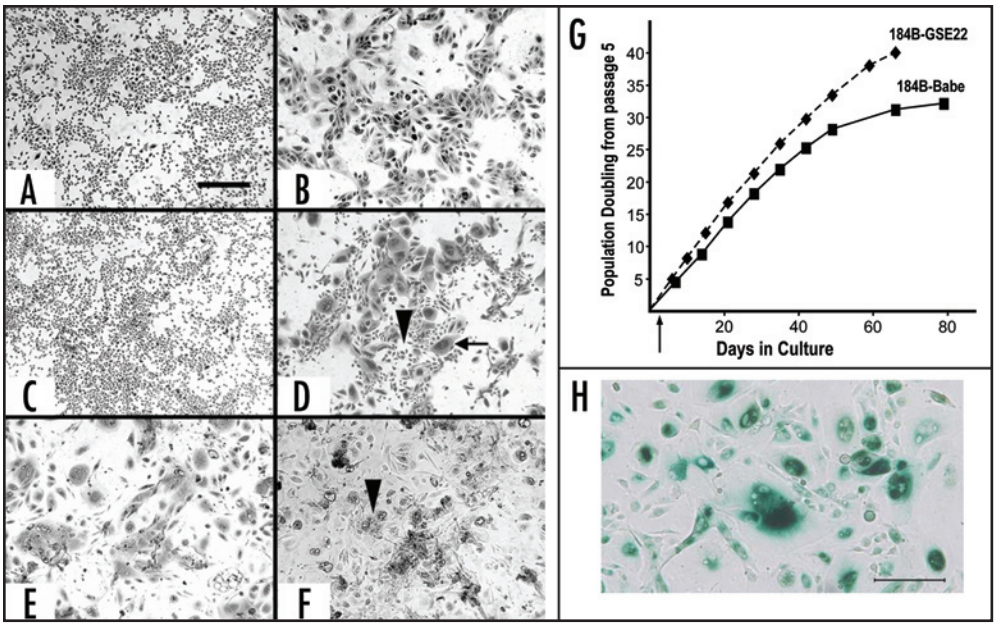

Figure 2. (A–F) Transduction of GSE22 leads to a crisis-like morphology rather than a mostly viable arrest in post-selection 184 HMEC. 184B HMEC infected with GSE22-containing or control (Babe) vectors at passage 5 were visually observed and photographed at subsequent passages. 184B-Babe (A) and 184B-GSE22 (C) at passage 7 show active growth of small cells with a cobblestone morphology. (B) 184B-Babe at agonescence, 2 months after plating at passage 15, contains mostly larger, flat cells with some vacuolization; the cell population can retain this morphology and viability for over a year. (D) 184-GSE22, two weeks after plating at passage 15, shows areas of small proliferating cells and many very large flat cells. (E) 184B-GSE22, two months after plating at passage 15, shows many large multi-nucleated vacuolated cells, cell debris, and some smaller cells. (F) 184B-GSE22, four months after plating at passage 15, shows mostly large multi-nucleated, vacuolated cells and abundant cell debris. The bar represents 200 microns. All photographs are at the same magnification. (G) Growth of 184B-Babe and 184B-GSE22 following transduction at passage 5 (arrow). (H) Post-selection 184B-GSE22 in crisis at passage 15 is SA-β-Gal(+).

Table 2 **Labeling Index (LI) of 184B-Babe and 184B-GSE22 at different passage levels**

	24 hr LI		4 hr LI	
Passage Number	**184B-Babe**	**184B-GSE22**	**184B-Babe**	**184B-GSE22**
8	93	93	39	42
12	40	76	6.9	30
13	17	70	3.2	20
14	16	44	3.7	19
15 (2 weeks)	15	43	2.3	16
15 (2 months)	5.6	25	1.9	15

be efficiently immortalized by hTERT,[28] indicating that HMEC do not need p53 inactivation to become immortal. To directly assess the role of p53 in enforcing stasis, primary 184F HMEC were grown in MM and transduced with the GSE22 or empty control vector at passage 2 or 3. Both cell populations showed similar growth rates and nearly complete growth arrest by passage 4, with expression of p16 and SA-β-Gal seen by immunohistochemistry in the large, senescent-appearing cells at passage 5 (Fig. 5B and C). As expected for HMEC grown in MM, no control cells showed escape from stasis; however, in two independent experiments a small number of clonal outgrowths appeared in the passage 5 GSE22-transduced cultures. Clonal outgrowths from one experiment ceased growth after an additional ~25 PD, with a morphology that resembled the post-selection 184B-GSE22 at crisis. Unlike post-selection HMEC, low levels of p16 expression were detectable in these populations (Fig. 5D). A clonal outgrowth from the second experiment maintained indefinite proliferative potential; this line has been called 184FGS1. These data indicate that in HMEC, p53 inactivation does not provide a proliferative advantage to prestasis populations as a whole. The very rare emergence of cells that overcame this first barrier suggests that these GSE22-transduced clones arose as a secondary, rather than a direct consequence of the loss of p53 function.

DISCUSSION

A variety of models and nomenclature have been employed in cultured human cell systems to describe senescence barriers; i.e., mechanisms that limit proliferative potential thereby precluding immortality. A commonly used model postulates two barriers, M1 or senescence, and M2 or crisis, that are both proposed to be consequences of shortened telomeres.[11,12] An M0 was later added to this model as a new name for the barrier we originally called selection.[41] More recently, telomere-length independent senescence barriers have been proposed.[1,2,13,15,17,40,42] These have been called senescence, extrinsic senescence, M1, M1.5, M^{INT}, and stasis. Other barriers to ongoing proliferation of finite lifespan cells have also been described, such as "stress-associated senescence" or "culture shock", due to sub-optimal culture conditions.[43,44] In most cases, these nomenclatures have not been defined and distinguished by specific molecular properties of the arrested cell populations. Cells are frequently called senescent based solely on their expression of SA-β-Gal, and a "senescent" (large, flat, vacuolated) morphology.

The data presented in this report, along with our long-term studies on HMEC, have led us to propose a simplified model and nomenclature for the senescence barriers encounter by cultured HMEC, based on expression of specific molecular properties (Fig. 6). Our model proposes that cultured HMEC encounter two mechanistically distinct senescence barriers: a stress-associated, telomere-length independent barrier, which we are calling stasis,[1] and a barrier due to ongoing telomere erosion leading to telomere dysfunction. Additionally, prestasis and post-selection finite lifespan HMEC in vitro are vulnerable to OIS, which induces a phenotype distinct from stasis and telomere dysfunction.[7]

We demonstrate here that the phenotype of the telomere dysfunction senescence barrier in HMEC depends upon whether or not p53 is functional. When p53 is functional, critically shortened telomeres produce a largely viable arrest, termed agonescence, due to the ability of p53 to respond to DNA damage by inducing cell cycle checkpoints. Thus, similar to what has been shown in in vivo mouse models, genomic instability based on telomere dysfunction

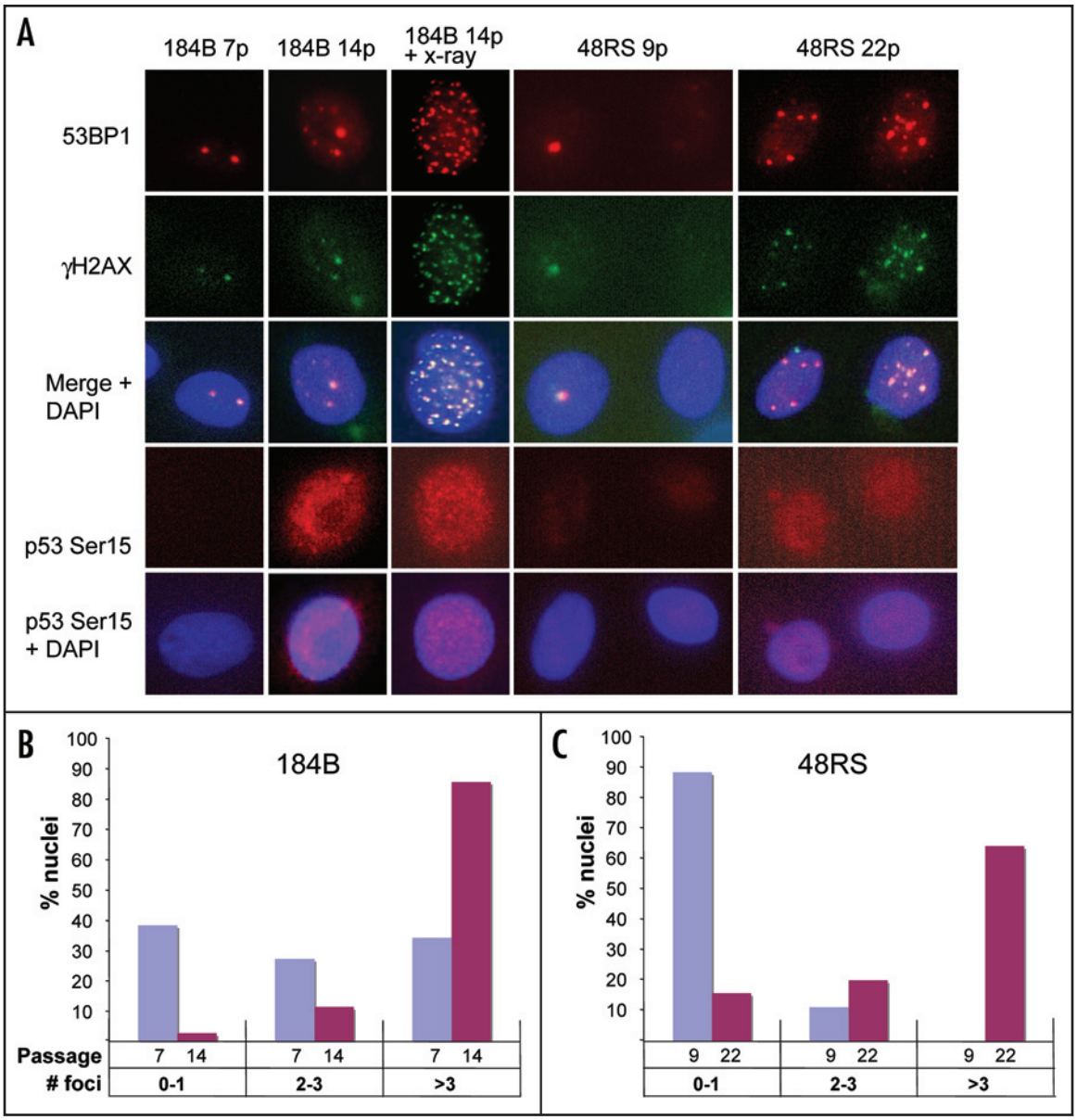

Figure 3. DNA damage responses in post-selection 184B and 48RS HMEC. (A) Representative fluorescent images of growing and agonescent post-selection HMEC, as well as x-ray irradiated HMEC, stained for p53 Ser15 (red), 53BP1 (red), phospho-histone H2AX (Ser 139) (green), and DNA (blue). Colocalization of the 53BP1 and phospho-H2AX signals is shown in yellow. (B)(C) Percentages of cells displaying 0-1, 2-3, or greater than 3 of the 53BP1 foci were calculated. For each cell population, at least five randomly selected fields were scored.

can trigger a restraining mechanism in the setting of intact p53;[45] such p53-mediated senescence mechanisms may pose a barrier to further malignant progression.[46,47] Cells which fail to arrest at agonescence die as a consequence of the genomic instability and mitotic failures produced by the critically shortened telomeres,[21] suggesting that p53 is unable to arrest all HMEC prior to acquisition of lethal or proliferation-inhibiting damage. Notably, virtually every metaphase spread examined in HMEC nearing agonescence showed gross chromosomal abnormalities, including numerous telomere associations.[21] When p53 function is abrogated in post-selection HMEC that have overcome stasis, the critically shortened telomeres produce crisis rather than agonescence; in the absence of p53-mediated checkpoint responses, virtually all the cells eventually die. Apoptosis is rare at telomere dysfunction, although it is higher during crisis than agonescence.[48] In our experiments, abrogation of p53 function by itself did not produce sustained reactivation of telo-merase activity or any immortal lines. In other reports,[49,50] rare immortalization was observed, likely due to the generation of an additional error or errors during the period of genomic instability occurring at crisis. Transduction of hTERT is sufficient to immortalize a variety of p53+ or p53- post-stasis human epithelial cell types,[28,40,51,52] further illustrating the telomere length dependence of agonescence and crisis. We have postulated that overcoming the telomere dysfunction barrier in post-selection HMEC requires generation of multiple errors that permit telomerase reactivation.[2]

In contrast with post-selection HMEC, we show here that GSE22-mediated abrogation of p53 function in early passage prestasis HMEC had no significant effect on growth of the population as a whole. Cells with and without p53 function ceased proliferation at stasis, associated with expression of p16 and SA-β-Gal, and a senescent

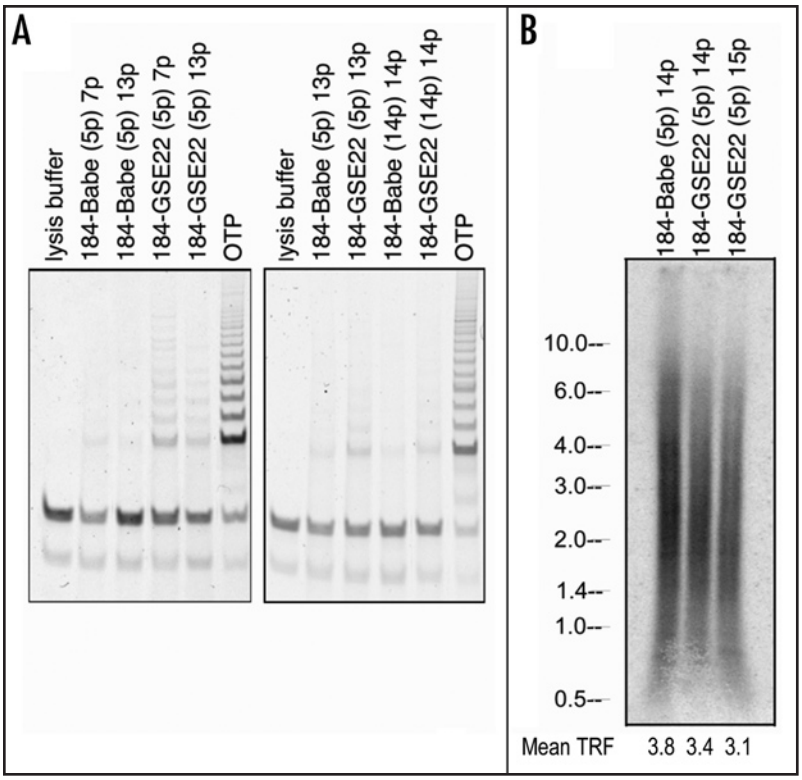

Figure 4. (A) Transduction of GSE22 in post-selection 184 HMEC does not produce significant, sustained reactivation of telomerase activity. 184B HMEC were transduced at passage 5 (5p) or 14 (14p) with GSE22 or control (Babe) vector, and assayed at the indicated passages for telomerase activity. (B). Mean TRF length of 184B-GSE22 at crisis is shorter than184B-Babe at agonescence. Genomic DNA was isolated from 184B-Babe HMEC at agonescence (14p) or from 184B-GSE22 HMEC during crisis (14p, 15p). Numbers on the left indicate the sizes of DNA molecular weight standards.

morphology. Similar results have been reported using HPVE6 inactivation of p53 function.[53] Thus p53 does not appear to enforce the initial proliferation barrier in cultured HMEC, in contrast to reports on other cell types such as human fibroblasts, keratinocytes, and astrocytes, where overcoming a first proliferation barrier has generally required loss of p53 function.[13,40,54] We propose that this difference is due to cell-type variations in stress responses.[55] Specifically, other cells may use the p53-dependent CKI p21 instead of or in addition to p16 to enforce an Rb-mediated stasis barrier. Ablation of p21 or Rb function can overcome this barrier even in the presence of functional p53.[56,57] In HMEC, p21 is not elevated at stasis,[21] and stasis can be efficiently overcome by introduction of an shRNA to p16 (Garbe J, Stampfer M, unpublished). We speculate that stresses that induce p53 may involve DNA damaging agents such as oxidative stress, and HMEC under routine culture conditions may be less susceptible to such damage than other cell types. In this regard, we have not seen any significant differences in long-term growth potential of prestasis HMEC when grown under 20% vs. 3% O_2 conditions (Garbe et al., in preparation). The absence of p53-dependent p21 induction enforcing stasis in cultured HMEC, along with the spontaneous silencing of p16 in rare HMEC grown in serum-free MCDB170 medium, presented an unusual situation that has facilitated distinguishing p53 input at the senescence barriers. It also permitted long-term growth of cultured finite lifespan HMEC (30–70 PD). These post-selection HMEC have been widely utilized, however we note that they have overcome the stasis barrier, and may possess significantly different properties and gene expression compared to prestasis HMEC derived from normal cells in vivo.[58-61]

Although p53 inactivation is not necessary to overcome stasis in HMEC, most studies with human cells have utilized agents that inactivate p53 to overcome a first proliferation barrier. Consequently, only crisis was observed at the telomere-length dependent senescence barrier in the p53(-) populations.[53,62] Since cells at agonescence are largely viable, SA-β-Gal(+), and express a senescent morphology, in the absence of additional molecular characterization, this telomere-length-dependent barrier may be equated with the viable stasis barrier. The assumption that HMEC at agonescence reflect M1/senescence led to the renaming of the earlier HMEC proliferation barrier, selection/stasis, as "M0";[41] however, our model and data indicate that no molecularly distinct "M0" exists. Rather we propose that agonescence, like M2/crisis, reflects a telomere dysfunction barrier, while stasis is similar to what has been called M1/senescence.

HMEC arrested at stasis are characterized by normal karyotypes, a low LI, viable arrest in G_1, elevated p16 levels, and a mean TRF >5 kb.[20,21,23] The cells also express SA-β-Gal, and have a senescent morphology. This molecular profile resembles what in many cultured cells has been called senescence, replicative senescence, or M1.[63] However, cellular diversity in stress responses, such as differences in sensitivity to oxidative stress-induced DNA damage, could generate variability in the phenotype seen at stasis. We suggest that what has been called stress-associated senescence due to "culture shock" also represents stasis; the greater the stress-inducing signals, the fewer PD prior to stasis. We have seen that the PD potential of cultured primary HMEC can vary from 15–60 PD, prior to a p16-associated arrest, depending upon culture conditions (Garbe et al., in preparation). An age-related increase in p16 expression is also reported for human breast, kidney, and pancreas tissues,[64-66] as well as rodent tissues,[67] suggesting that stress-induced responses may occur in vivo.

Cultured human fibroblasts commonly proliferate for more PD than epithelial cells before encountering a senescence barrier. The molecular profile of most fibroblasts called senescent contains properties more similar to those defining HMEC stasis than agonescence,[63] and overcoming this barrier by inactivation of p16/RB and p21/p53 function leads to crisis.[15,56] Some fibroblasts strains, particularly those with reduced p16 expression such as BJ, may display >80 PD in culture, and cells in such populations could encounter agonescence prior to stasis. Unlike most fibroblast strains, BJ populations at proliferative arrest exhibit karyotypic abnormalities in a minority of the cells; however most cells did not exhibit telomeric end-associations.[68] This is distinct from HMEC at agonescence, where virtually all cells showed gross chromosomal abnormalities and telomere associations,[21] indicating that at least for HMEC, telomere dysfunction does not produce a p53-mediated senescence arrest prior to the formation of gross chromosomal aberrations.

To have terminology tied to specific molecular criteria, we propose the model shown in Figure 6. The non telomere-length dependent, stress-associated senescence barrier is called stasis,[1,69] while the telomere-length dependent senescence barrier is referred to as either agonescence (when p53 is functional),[24] or crisis (when p53-dependent functions are absent). Stasis is characterized by elevated

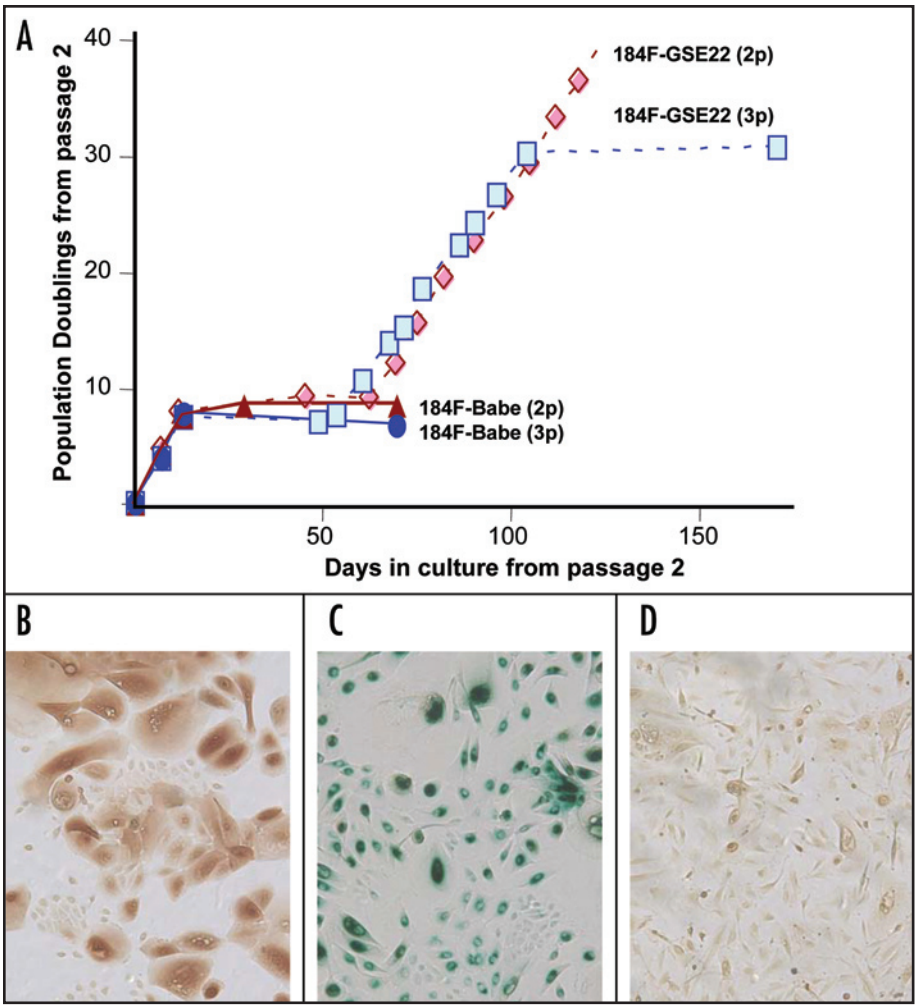

Figure 5. Transduction of GSE22 into prestasis 184F HMEC does not overcome stasis. 184F HMEC were transduced at passage 2 (2p) or 3 (3p) with GSE22 or control (Babe) vector, and proliferative potential determined. (A) All populations showed cessation of most growth by passage 5, however rare colonies appeared in the GSE22-transduced cultures that maintained growth until either a crisis-like arrest (184F-GSE22 3p) or emergence of an immortal line (184F-GSE22 2p). (B) 184F-GSE22 cells at passage 5 with a senescent morphology are p16(+) and (C) SA-β-Gal(+). (D) 184F-GSE22 (3p) shows weak staining for p16 at 9p.

levels of the CKIs p16 and/or p21, a low LI, G_1 arrest, and largely normal karyotypes; it can also be readily overcome by multiple types of errors that inactivate an Rb-mediated barrier. Agonescence is characterized by a moderate LI, mostly viable arrest at all phases of the cell cycle with some cell death, critically shortened telomeres, and widespread karyotypic abnormalities. Crisis is characterized by a high LI, widespread karyotypic abnormalities, and eventual massive cell death. The properties associated with Raf-1 induced OIS in HMEC differ from what is seen for stasis or telomere dysfunction.[7] Generic usage of the term "senescence" to refer to both telomere length-independent stasis, and barriers due to telomere dysfunction, may obscure distinctions important for understanding human cellular aging, immortalization, and carcinogenesis. For example, cultured rodent cells, which readily spontaneously immortalize, lack stringent repression of telomerase activity and may contain long telomeres.[70] What has been called senescence in rodent cells may most closely resemble Rb-mediated non telomere-length dependent stasis. Senescence in mouse embryo fibroblasts can be reversed by inactivation of RB, even in the presence of functional p53.[71]

In contrast, in long-lived organisms such as humans, stringent telomerase repression eventually leads to telomere erosion and the telomere dysfunction senescence barrier, even in cells that have overcome stasis. The genomic instability induced by telomere dysfunction is not readily reversible, and overcoming this barrier by reactivation of telomerase requires rare errors.[2] Unlike rodent cells, spontaneous transformation to immortality of human cells cultured from normal tissues is virtually nonexistent. Thus the senescence barrier responsible for enforcing stringent mortality in cultured human cells is telomere length dependent. Given the importance of senescence barriers as tumor suppressor mechanisms, as well as the potential clinical utility of markers of senescence,[14,16] use of molecularly defined nomenclature for senescence barriers may facilitate our understanding of and therapeutic approaches to human carcinogenesis.

Acknowledgements

We thank Gerri Levine for excellent technical assistance, Kathy Bjornstad and Ellie Blakely for assistance with microscopy, and Drs. Andrei Gudkov, Peter Chumakov, Christian Beausejour and Judy Campisi for reagents. This work was supported by NIH grant CA24844, Department of Defense BCRP grant W81XWH-04-1-0580 (M.R.S., J.G.), the Office of Energy Research, Office of Health and Biological Research, U.S. Department of Energy under Contract No. DE-AC03-76SF00098 (M.R.S.), NIH grant CA73952 (T.T.), Howard Hughes Medical Institute Pre-Doctoral fellowship (C.H.), and Ruth L. Kirschstein National Research Service Award Individual Fellowship CA108480-03 (EB).

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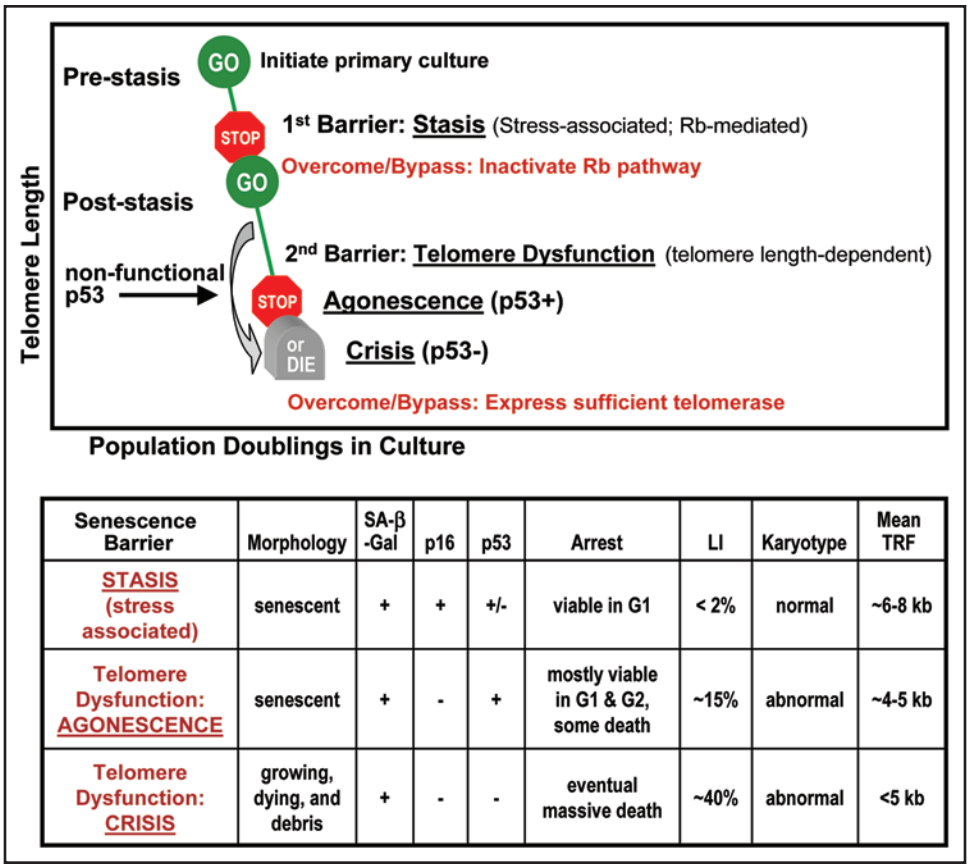

Senescence Barrier	Morphology	SA-β -Gal	p16	p53	Arrest	LI	Karyotype	Mean TRF
STASIS (stress associated)	senescent	+	+	+/-	viable in G1	< 2%	normal	~6-8 kb
Telomere Dysfunction: AGONESCENCE	senescent	+	-	+	mostly viable in G1 & G2, some death	~15%	abnormal	~4-5 kb
Telomere Dysfunction: CRISIS	growing, dying, and debris	+	-	-	eventual massive death	~40%	abnormal	<5 kb

Figure 6. Molecularly defined model for senescence barriers in cultured HMEC. This model postulates at least two mechanistically distinct barriers to indefinite proliferation in cultured HMEC. The non telomere-length dependent stasis barrier is enforced by the CKI p16. In other cell types, p53-dependent p21 expression may also enforce Rb-mediated stasis. p53-inducing stresses cells could produce distinct phenotypes from what is seen in the cultured HMEC at stasis. HMEC in vivo, or in response to DNA-damaging stresses in vitro such as radiation, might also employ p53-dependent p21 to enforce stasis. Multiple types of errors that can inactivate a stress-induced Rb-mediated barrier can overcome stasis. Many such errors (e.g., p16 silencing/mutation, RB and p53 mutations/inactivation, cyclin D1 or cdk4 overexpression) are found in early stage human carcinoma development, and could give rise to clonal hyperplastic outgrowths. The phenotype of the telomere dysfunction barrier depends upon whether or not p53 is functional. We have postulated that overcoming the telomere dysfunction barrier is an extremely rare event because it requires multiple errors to reactivate telomerase.[2] HMEC nearing arrest from telomere dysfunction have some properties similar to premalignant lesions such as DCIS, e.g., very short telomeres and genomic instability; reactivation of telomerase activity can also be detected in DCIS tissues. The phenotype of HMEC subjected to OIS is distinct from both stasis and agonescence, e.g., unlike some other cell types, there is no requirement for either p16 or p53 function for OIS in HMEC, arrest is at all phases of the cell cycle, and telomeres are not critically shortened.[7]

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