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Authors
Bazarov, AV
Lee, WJ
Bazarov, I
et al.

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The Specific Role of pRb in p16\textsuperscript{INK4A} Mediated Arrest of Normal and Malignant Human Breast Cells

Alexey V. Bazarov\textsuperscript{1,2}, Won Jae Lee\textsuperscript{2}, Irina Bazarov\textsuperscript{2}, Moses Bosire\textsuperscript{2}, William C. Hines\textsuperscript{2}, Basha Stankovich\textsuperscript{2}, Agustin Chicas\textsuperscript{3}, Scott W. Lowe\textsuperscript{3}, and Paul Yaswen\textsuperscript{2}

\textsuperscript{1}Department of Medicine, University of California, San Francisco, CA, USA
\textsuperscript{2}Life Sciences Division, Lawrence Berkeley National Laboratory, Berkeley, CA, USA
\textsuperscript{3}Cancer Biology and Genetics Program, Sloan-Kettering Institute for Cancer Research, New York, NY, USA

Correspondence to: Paul Yaswen, Department of Cancer and DNA Damage Responses, Lawrence Berkeley National Laboratory, Mailstop 977, 1 Cyclotron Rd., Berkeley, CA 94720, USA. Tel.: +1 (510) 486-4192; fax: +1 (510) 486-5586; e-mail: P_Yaswen@lbl.gov

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Abbreviations: CDK, cyclin dependent kinase; p16, \textit{CDKN2A} gene product; HMEC, human mammary epithelial cell; p21, \textit{CDKN1A} gene product; p107, \textit{RBL1} gene product; p130, \textit{RBL2} gene product; pRb, retinoblastoma gene product;
Abstract

RB family proteins, pRb, p107, and p130, have similar structures and overlapping functions, enabling cell cycle arrest and cellular senescence. pRb, but not p107 or p130, is frequently mutated in human malignancies. In human fibroblasts acutely exposed to oncogenic ras, pRb has a specific role in suppressing DNA replication, and p107 or p130 cannot compensate for the loss of this function; however, a second p53/p21-dependent checkpoint prevents escape from growth arrest. This model of oncogene-induced senescence requires the additional loss of p53/p21 to explain selection for preferential loss of pRb function in human malignancies. We asked whether similar rules apply to the role of pRb in growth arrest of human epithelial cells – the source of most cancers. In two malignant human breast cancer cell lines, we found that individual RB family proteins were sufficient for the establishment of p16-initiated senescence, and that growth arrest in G1 was not dependent on the presence of functional pRb or p53. However, senescence induction by endogenous p16 was delayed in primary normal human mammary epithelial cells with reduced pRb, but not with reduced p107 or p130. Thus under these circumstances, despite the presence of functional p53, p107 and p130 were unable to completely compensate for pRb in mediating senescence induction. We propose that early inactivation of pRb in pre-malignant breast cells can, by itself, extend proliferative life span, allowing acquisition of additional changes necessary for malignant transformation.
**Introduction**

The stable cell cycle arrest that occurs during the senescence response to a variety of stressful stimuli including oncogene induction, DNA damage, and critically short telomeres\(^1,2\), has been shown to serve as a key impediment to the development of invasive cancers\(^3-5\). This growth arrest is often mediated through RB family proteins (pRb, p107 and p130), which have similar structures and overlapping functions\(^6,7\). RB family proteins are regulated by cyclin dependent kinases (CDKs) 2, 4, and 6\(^8\). When CDK activity is inhibited by the tumor suppressor p16\(^{INK4A/CDKN2A}\) (p16), hypophosphorylated RB family proteins form complexes with E2F transcription factors, leading to cell cycle arrest\(^9\). Despite the similarities among RB family proteins, defects in pRb, but not in p107 or p130, have been associated with several human cancers\(^10,11\). This suggests that pRb has unique tumor suppressor properties that are not attributable to p107 or p130. In support of this concept, pRb was recently shown to be preferentially associated with E2F targets involved in DNA replication during ras-induced cellular senescence of primary human fibroblasts\(^12\). In these experiments, suppression of pRb, but not p107 or p130, allowed continued DNA synthesis. While these results indicate a special function of pRb, manifestation of a selective growth advantage by fibroblasts lacking pRb expression also required prior inactivation of a second p53/p21-dependent checkpoint. In the current work, we asked whether pRb has non-redundant tumor suppressor functions in epithelial cells - the precursors of a majority of human cancers, and if the phenotypic consequences of pRb loss require a particular genetic background.
Results

Individual RB family proteins can compensate for each other in mediating growth arrest of human breast cancer cell lines. To directly investigate the RB family protein requirements for p16-initiated growth arrest in human epithelial cells, we utilized two model systems. The first model employed exogenously introduced tetracycline-regulated p16 genes in MDA-MB-231 (estrogen receptor α (-), p53(-)) and MCF7 (estrogen receptor α (+), p53(+)) human breast cancer cell lines, both which lack endogenous p16 gene function. We created stable sub-lines in which pRb, p107, or p130 were individually suppressed through the action of shRNA-encoding retroviruses. While the shRNAs each effectively reduced protein levels, none of them prevented G1 arrest when p16 was induced by the addition of doxycycline. Thus, in contrast to human fibroblasts undergoing ras-induced senescence, human breast cancer cells undergoing acute p16-induced senescence did not specifically require the presence of pRb to suppress DNA replication. In addition, extended exposure to p16 led to similar losses in colony forming efficiency by control cells and cells in which the expression of individual RB family proteins was suppressed. Interestingly, the induction of growth arrest by p16 in cells lacking pRb expression was not dependent on the presence of functional p53, as MDA-MB-231 cells express mutated, non-functional p53.

To investigate the possibility that p16 causes growth arrest in these cells via an RB family protein independent pathway, we attempted to knock down all three RB family proteins simultaneously by introducing a single vector encoding shRNAs against them. However, despite multiple rounds of infection, we were not able to efficiently suppress all three proteins in the breast cancer cell lines using this vector (data not shown). In an alternative approach, we
employed human papilloma virus (HPV) oncoprotein E7, which binds and inactivates all three RB family proteins\textsuperscript{15}. HPV E7 expression partially prevented p16-initiated G1 growth arrest in MDA-MB-231 cells (Fig. 1D). In contrast, expression of HPV E6, which inactivates p53, but does not interact with RB family members\textsuperscript{15}, had no effect on p16-initiated G1 growth arrest. These results collectively suggest that in at least some breast cancer cells: a) RB family proteins are required for p16-initiated growth arrest, b) individual RB family proteins are sufficient to mediate growth arrest, and c) functional p53 is not required for p16-initiated growth arrest in the presence or absence of pRb.

**Suppression of pRb, but not p107 or p130, extends the proliferative lifespan of primary human mammary epithelial cells.** We further investigated the roles of RB family proteins using primary normal human mammary epithelial cells (HMECs) as a model. Propagation of primary HMECs in conventional adherent cultures is accompanied by spontaneous induction of endogenous p16, which leads to senescence and cessation of net population increases after approximately ten population doublings\textsuperscript{16}. Suppression of p107, p130, or both did not lead to extension of HMEC proliferative lifespan in two independent experiments using different specimens (Figs. 2A & B, and S2A & B). In contrast, suppression of pRb in HMEC cultures allowed these cells to proliferate significantly longer than controls, although the shpRb-transduced HMEC cultures still ultimately underwent senescence. Combining shRNAs against p107 or p130 with those against pRb led to small additional increases in proliferative lifespans. During the prolonged proliferative phase, shpRb-transduced HMEC cultures were morphologically heterogeneous, containing mixtures of large senescent and small actively growing cells (Fig. 2C), whereas shpRb/107/130-transduced HMEC cultures proliferated rapidly,
without exhibiting morphological heterogeneity. The appearance and proliferation rates of the latter cells resembled those of cells expressing p16 shRNAs. Thus, although p107 and/or p130 retained some ability to compensate for pRb in mediating p16-initiated growth arrest of primary HMECs, the compensation was inefficient.

We attempted to determine whether knocking down pRb conferred unique properties that might be responsible for the increased life span of shpRb-transduced HMEC cultures. In pRb-deficient fibroblasts transfected with Ras oncogenes, increased levels of cyclin E expression were linked to increased DNA replication, however pRb loss triggered a second p53/p21-dependent checkpoint that prevented escape from senescence \(^{12}\). In contrast, while we observed significantly elevated levels of both p16 and p21 CDK inhibitors in shRb-transduced HMECs, there were no corresponding increases in cyclin E levels that might compensate and thereby explain the ability of the shpRb-transduced HMECs to continue growth under these conditions (Figs. 2D and S2C). Interestingly, a faster migrating form of p21 was retained in all the shRNA-expressing HMECs with the exception of those expressing p16 shRNAs. The significance of this altered form of p21 - the presence of which did not correlate with growth rate or proliferative potential, is not known, but may correspond to a truncated version that lacks a nuclear localization signal in the N-terminal region of the protein \(^{17}\).

**The different abilities of RB-family proteins to compensate for each other in breast tumor cell lines versus HMECs are unlikely to be solely due to the levels of CDK4/6 inhibition achieved.** Since the canonical role of p16 is inhibition of CDK 4/6 activity, we asked whether the different abilities of RB-family proteins to compensate for each other in breast tumor cell lines
versus HMECs was related to the level of CDK inhibition achieved. We reasoned that in the presence of sufficient p16 (or when CDK4/6 activity is maximally inhibited), as is likely in the breast cancer cells expressing exogenously introduced tet-inducible p16 genes, p107 and/or p130 have sufficient residual compensatory activity to block DNA replication and cell proliferation; however, when p16 is limiting (or CDK4/6 activity is only partially inhibited), as is likely in HMECs in which endogenous p16 is slowly induced, p107 and/or p130 are less efficient at blocking DNA replication and proliferation than pRb. To test this possibility, we assessed the relative susceptibilities of MDA-MB-231 cells expressing shRNAs against different RB family proteins to growth inhibition by PD 0332991, a specific small molecule inhibitor of CDK4/6 \(^\text{18}\). These assays showed that the cells expressing shRNAs against pRb had slightly higher IC\(_{50}\) values than cells expressing the control vector or shRNAs against p107 or p130, but the differences were not significant (Fig. 3). Thus the different abilities of RB-family proteins to compensate for each other in breast tumor cell lines versus HMECs are unlikely to be solely due to the levels of CDK4/6 inhibition achieved.

**Discussion**

We have employed retrovirally transduced shRNAs to determine whether stable growth arrest associated with p16-initiated senescence in human breast epithelial cells is mediated through one or more RB family proteins. Despite pRb, p107, and p130 knockdowns of >90% in one model employing MCF7-tet-p16 and MDA-MB-231-tet-p16 breast cancer cell lines stably expressing shRNAs against each of the individual RB family proteins, p16 induction still resulted in irreversible G1 growth arrest in each case. This finding suggests that there is some redundancy in the ability of the individual RB family proteins to mediate irreversible growth arrest. Cases of
functional redundancy within this gene family have been reported in a number of murine and human cell types \(^{19,20}\). Our finding that expression of HPV E7, which inactivates all 3 RB family proteins, prevents p16-induced growth arrest in breast cancer cells, is consistent with the concept that at least one RB family protein must be present and functional for growth arrest to occur. Additional support comes from our studies of primary HMECs, in which we found that, unlike the case for individual knockdowns, simultaneous downregulation of all three RB family proteins enabled these cells to efficiently overcome p16-mediated senescence. This finding may explain why aberrations in upstream regulators such as p16, cyclin D1, and CDK4, which presumably affect the regulation of all 3 RB family proteins simultaneously, are more common than aberrations in the individual RB family proteins themselves in breast cancers. From a clinical standpoint, it is encouraging that even aggressive cancer cells such as MDA-MB-231-sh-pRb, lacking both p53 and pRb tumor suppressors, are susceptible to induction of irreversible senescence. This suggests that therapies employing small molecule inhibitors of CDK4/6 may be effective even in some tumors lacking functional pRb.

Interestingly, the cancer cell lines in which pRb expression was suppressed showed no resistance to p16-initiated growth arrest, while HMECs in which pRb expression was suppressed exhibited a clear proliferative advantage despite increases in the levels of endogenous p16. The difference in the ability of RB family proteins to compensate for one another in the different experimental systems does not appear to be due simply to the level of CDK inhibition achieved, as cancer cells with suppressed pRb displayed susceptibility to short-term growth inhibition by a small molecule CDK inhibitor similar to that of pRb-competent cells. It is possible that the differing responses to pRb depletion may result from differences in the abundance and availability of distinct E2F
factors in malignant versus non-malignant cells. In particular, the levels of E2F1 mRNA have been found to be reduced in malignant compared to normal breast tissues. Altered stoichiometry, along with stress-induced post-translational modifications and changes in localization may limit the available E2F1, permitting tighter regulation by p130 and p107 in the absence of pRb.

While the exact reason for the distinctive role of pRb in establishing senescence in normal epithelial cells remains to be established, our results suggest why pRb may be preferentially inactivated during breast cancer progression. Whereas suppression of p107 and/or p130 expression does not affect the survival or growth of normal mammary epithelial cells, inactivation of pRb makes these cells partially resistant to p16-induced senescence, enabling them to survive increased levels of stress. An expanded ability to survive and proliferate under stressful conditions (e.g., oncogene induction, DNA damage, or critically short telomeres) could allow pRb-negative HMECs to acquire additional genetic and epigenetic changes leading eventually to malignant transformation.

Materials and Methods

Cells and retroviruses. MDA-MB-231 and MCF7 cells were obtained from the American Type Culture Collection (ATCC) and cultured in DMEM medium (Invitrogen) with 10% fetal bovine serum (Tet System Approved FBS, Clontech). Primary HMECs were obtained from histologically normal reduction mammoplasty tissues (Cooperative Human Tissue Network) and grown in MEGM medium (Lonza). Inducible expression of p16 was accomplished by inserting a p16 cDNA in front of the doxycycline-inducible promoter in the pLenti CMV/TO Neo DEST
(685-3) vector \(^{25}\), and introducing this vector into cells expressing high levels of tetracycline responsive repressor (tet-R) protein. Retroviruses encoding pRb, p107, and p130 shRNAs were generated in the LMP vector (Thermo Scientific) as previously described \(^{12}\). Retroviruses encoding HPV16 E6 or E7 genes in the LXSN vector were harvested from stable producer cell lines \(^{26}\).

**Immunoblotting.** Total protein lysates were collected and processed for immunoblotting by standard methods. Antibodies used included those against p16 (Ab-1; NeoMarkers), p21 (CP74, Millipore), pRb (554136, BD Pharmergen), p107 (sc-318, Santa Cruz Biotechnology), p130 (sc-317, Santa Cruz Biotechnology), cyclin E (sc-481, Santa Cruz Biotechnology), and actin (C4, Millipore).

**Microscopy.** Cells were viewed and photographed using a phase contrast Nikon Eclipse TS100 microscope (Diagnostic Instruments), and images were processed using SPOT 4.1 software (Diagnostic Instruments).

**Quantitative RT-PCR.** Total RNAs were prepared using RNA-EZ kits (Qiagen), and cDNAs were synthesized using iScript cDNA synthesis kits (Bio-Rad). Quantitative RT-PCR reactions were performed using SYBR GreenER kits (Invitrogen) and an iCycler with a MyIQ optical unit (Bio-Rad). The following primers were used:

- **TBP** - CACGAACCACGGCACTGATT (F), TTTTCTTGCTGAGTGCTGGAC (R);
- **pRb** - AAAGGGAGAATGCCTCCTGGACCT (F), TTAGCCTGTGCTGTCAGTTTCCCT (R);
- **p107** - AAAGGAGAATGCCTCCTGGACCT (F), TTAGCCTGTGCTGTCAGTTTCCCT (R);
- **p130** - AACGCTGGTTCAGGAACAGAGACT (F), AGAAACTGGAGTCACACAAGGGCT (R);
- **p16** - AGCATGGAGCTTCCGGCTGACT (F), ATCATCATGACCTGGATCGGCCT (R).
Cell cycle analyses. Cells were dissociated with trypsin, washed with cold phosphate buffered saline (PBS), then resuspended in 0.5 ml ice cold PBS and fixed by drop-wise addition of 4.5 ml of cold 70% ethanol. The cells were then washed with PBS, resuspended in staining solution (PBS, 0.1% Triton X-100, 0.2 mg/ml DNase-free RNase A (R-6513, Sigma), 0.02 mg/ml propidium iodide (P-4170, Sigma)), incubated for 30 min at room temperature, then kept at 4°C until assayed. Flow cytometry was performed using a FACSCalibur instrument (Becton Dickinson) and results were analyzed using FlowJo 9.3.3 software.

Proliferation assays. Growth inhibition by PD0332991 was determined as described. Briefly, cells were seeded in triplicate at 5,000 cells per well in 24-well plates. After 24 hours, they were treated with 7 concentrations of PD 0332991 (1000, 500, 250, 125, 62.5, 31.25, and 0 nM) in growth medium. After a 6-day incubation, the cells were harvested and counted, and dose-response curves were generated. Three independent experiments were performed, and IC_{50} values were compared using Student’s t-test (http://www.physics.csbsju.edu/stats/t-test_bulk_form.html).

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preferentially inhibits proliferation of luminal estrogen receptor-positive human breast
Figure Legends

**Figure 1.** Individual RB family proteins can compensate for each other in mediating p16-initiated growth arrest of MDA-MB-231 human breast cancer cells. (A) MDA-MB-231-TETp16 cells stably transduced with retroviruses encoding shRNAs against indicated RB family proteins were harvested and analyzed by immunoblotting. Prominent Ponceau S stained bands were used as loading controls. (B) Cells transduced with indicated shRNAs or vector alone were treated with 1 μM doxycycline for 48 hours, then harvested and subjected to propidium iodide (PI) staining and flow cytometry. Exponentially growing cells transduced with an empty vector were used as controls. (C) Cells transduced with indicated shRNAs or vector alone were treated with 1 μM doxycycline for 48 hours, then dissociated, counted, and re-plated at clonal densities in the absence of doxycycline. After 3 weeks, the resulting colonies were counted and plating efficiency was calculated as (colonies formed/cells plated) x 100%. Relative plating efficiencies were then calculated by comparison to un-induced controls. Mean values and standard deviations (n=3) are shown. (D) MDA-MB-231-TETp16 cells expressing HPV E6 or HPV E7 were either left untreated (-DOX) or treated with 1 μM doxycycline for 48 hrs (+DOX). The cells were then harvested, subjected to PI staining and analyzed by flow cytometry.

**Figure 2.** Suppression of pRb, but not p107 or p130, extends the proliferative lifespan of primary human mammary epithelial cells. (A) The relative expression of mRNAs encoding pRb, p107 and p130 was measured by qRT-PCR for actively growing HMECs stably transduced with retroviruses encoding shRNAs against the indicated proteins. Quantitative results were normalized to those of a stably expressed reference transcript encoding TATA binding protein (TBP). Mean values and SD (n=3) are shown. (B) Total population doublings of HMECs stably
transduced with retroviruses encoding shRNAs against the indicated proteins are plotted versus time. (C) Representative morphologies of HMEC cultures expressing the indicated shRNAs (200X). The micrographs were taken 30 days (Vector, shp107, shp130), 52 days (shpRb), 42 days (shpRb/107/130), or 23 days (shp16) following viral transduction. (D) Total levels of cyclin E, p21, and p16 proteins were compared by immunoblotting in HMECs transduced with indicated shRNAs. Actin abundance was used as an indication of loading equivalence. The results shown are representative of results obtained using HMECs from two independent specimens. See additional results in Figure S2.

**Figure 3.** Cells expressing shRNAs against pRb were slightly less sensitive than cells expressing shRNAs against p107 or p130 to growth inhibition by a specific CDK inhibitor. The bar graph indicates PD 0332991 mean IC$_{50}$ values (nM) for MDA-MB-231 derived cell lines transduced with shRNAs against indicated RB-family proteins. Standard deviations (n=3) along with $p$ values (Student’s $t$-test) are shown.

**Supplemental Figure Legends**

**Figure S1.** Individual RB family proteins can compensate for each other in mediating p16-initiated growth arrest of MCF7 human breast cancer cells. (A) MCF7-TETp16 cells stably transduced with retroviruses encoding shRNAs against indicated RB family proteins were harvested and analyzed by immunoblotting. Prominent Ponceau S stained bands were used as loading controls. (B) MCF7-TETp16 cells transduced with indicated shRNAs or vector alone were treated with 1 mM doxycycline for 48 hours, then harvested and subjected to propidium
iodide (PI) staining and flow cytometry. Exponentially growing cells transduced with an empty vector were used as controls.

**Figure S2.** Suppression of pRb, but not p107 or p130, extends the proliferative lifespan of primary human mammary epithelial cells from a second specimen. (A) The relative expression of mRNAs encoding pRb, p107, and p130 was measured by qRT-PCR for actively growing HMECs stably transduced with retroviruses encoding shRNAs against the indicated proteins. Quantitative results were normalized to those of a stably expressed reference transcript encoding TATA binding protein (TBP). Mean values and SD (n=3) are shown. (B) Total population doublings of HMECs stably transduced with retroviruses encoding shRNAs against the indicated proteins are plotted versus time. (C) Total levels of cyclin E, p21, and p16 proteins were compared by immunoblotting in HMECs transduced with indicated shRNAs. Actin abundance was used as an indication of loading equivalence.