

E2F-1 is a critical modulator of cellular senescence in human cancer

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Abstract. Senescence limits cellular proliferation, and therefore might be a mechanism which could suppress the progression of cancer. Herein we show that E2F1, a transcription factor essential to a cell cycle progress and a main target of tumor suppressor Rb, is a critical barrier for the induction of senescence. Human cancer cells transfected with siE2F1 were shown to express replicative senescence markers, in addition to yielding positive results upon SA- β -Gal staining. Consistent with the notion of the critical role in senescence of E2F1, cells which overexpressed E2F1 proved to be immune to the induction of senescence. Importantly, it appears that E2F1 depletion-induced cancer cell senescence is not reliant on the integrity of either Rb or p53. Our results provide a molecular explanation for the selectivity with which senescence induction occurs, and also provides insights into the possibility of using E2F1 as a therapeutic target in the treatment of cancer.

Introduction

Cellular senescence is a stable form of cell cycle arrest which limits the proliferation of damaged cells, and might be one of the foci of a strategy to effect the suppression of tumorigenesis (1). Replicative senescence is known to be an irreversible process, in which phenotypic changes including growth arrest, apoptosis resistance, and altered differentiation are induced (2,3). Mutations in certain tumor suppressor genes have been shown to compromise senescence, which may contribute to both cell immortalization and cancer (4-6). The senescence response to DNA damage and oncogene over-expression is modulated by Rb and p53 (7-12). Therefore, defects in the process of cellular senescence may contribute to drug resistance in cancer chemotherapy (13,14). Members of

the Rb family are known to function as corepressors of the E2F transcription factors, and are also known to recruit histone deacetylases (HDACs) to E2F1-dependent promoters, thereby effecting the repression of gene expression. Previous studies have reported that p107 and p130, but not Rb, are strongly associated with E2F-responsive genes during the cell cycle and during quiescence (15,16). However, Narita *et al* have identified senescence-associated heterochromatic foci (SAHF) as distinct senescence-specific chromatin structural formations, involving the recruitment of heterochromatin proteins and Rb to E2F-responsive promoters, which have been previously associated with the stable repression of E2F target genes (17). Consistent with the notion that Rb and p53 play crucial roles in this process, p16INK4a and p21 have also been shown to induce the senescence phenotype in normal human fibroblasts (18).

E2F1 is known to regulate the progression of the cell cycle via the activation of transcription in several genes required for DNA synthesis. In addition to this transactivation activity, E2F1 functions in the binding and modulation of unphosphorylated Rb, cyclin A, and Mdm2 (19-24). Ectopic E2F1 expression induces DNA synthesis (25-28), and neoplastic transformation (19,20,23), and also increases tumorigenesis in p53-deficient mice. Conversely, the germ line inactivation of E2F1 results in an increase in tumorigenesis in mice (29,30). Previously, E2F1 was shown to induce a senescence-like phenotype in normal human fibroblasts, under the condition that E2F1 and p53 were both active (31).

We provide clear evidence that E2F1 plays a pivotal role in the suppression of senescence. We show that E2F1 depletion induces the senescence phenotype in tumor cells, and that this process occurs independently of both p53 and Rb. We also demonstrate that the ectopic expression of E2F1 inhibits the progression of cellular senescence. Together, our results indicate, for the first time to our knowledge, that E2F1 may be a critical factor for the progression of tumors in the presence or absence of p53 or Rb.

Materials and methods

Reagents and vectors. Cell lines including human prostate carcinoma DU145, LNCaP and PC3 cells were purchased from American Type Culture Collection (Rockville, MD). RPMI-1640 and fetal bovine serum were obtained from Gibco Life Science. Doxorubicin, 3-(4,5-dimethyl-2-thiazolyl)

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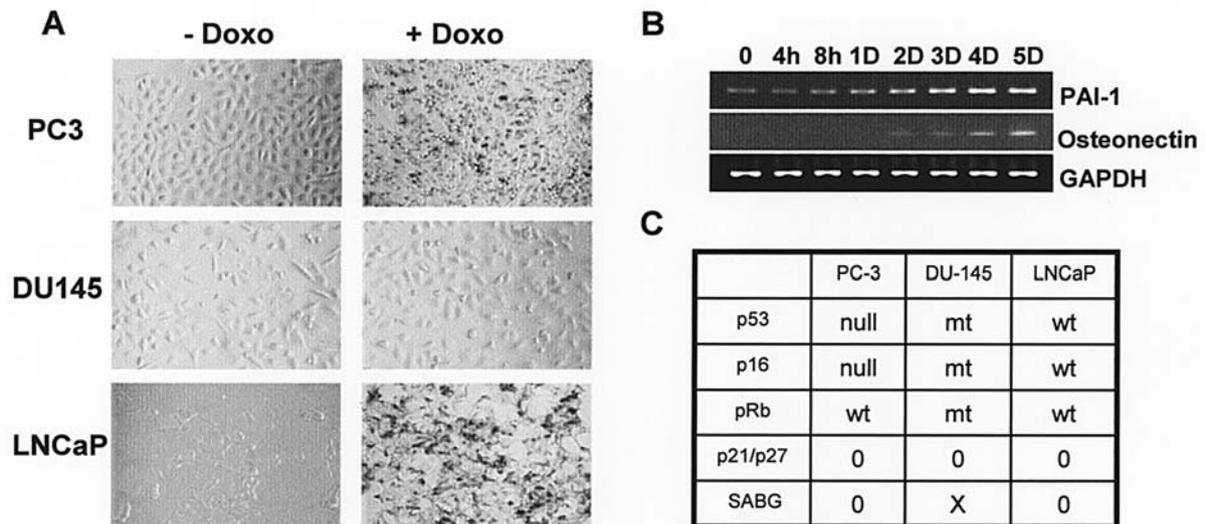


Figure 1. Differential senescence in DNA damage-induced human prostate cancer cells PC-3, DU145 and LNCaP. Cells were seeded at a concentration of 2×10^5 cells/ml of medium, then left to attach for 24 h before being exposed to 10 nM doxorubicin. At the indicated time intervals thereafter, the PC-3 cells were harvested for Western blot analysis. A, Typical SA- β -gal staining in PC-3 and LNCaP, but not in the DU145 cells. B, Analysis of changes in senescence-induced gene expression in the PC-3 cells which had received doxorubicin treatment for the indicated intervals. The total RNAs were prepared from the cells, and were subject to RT-PCR with the primers shown on the right. β -actin was used as a loading control. C, The status of senescence-associated genes in the three human prostate cancer cell lines.

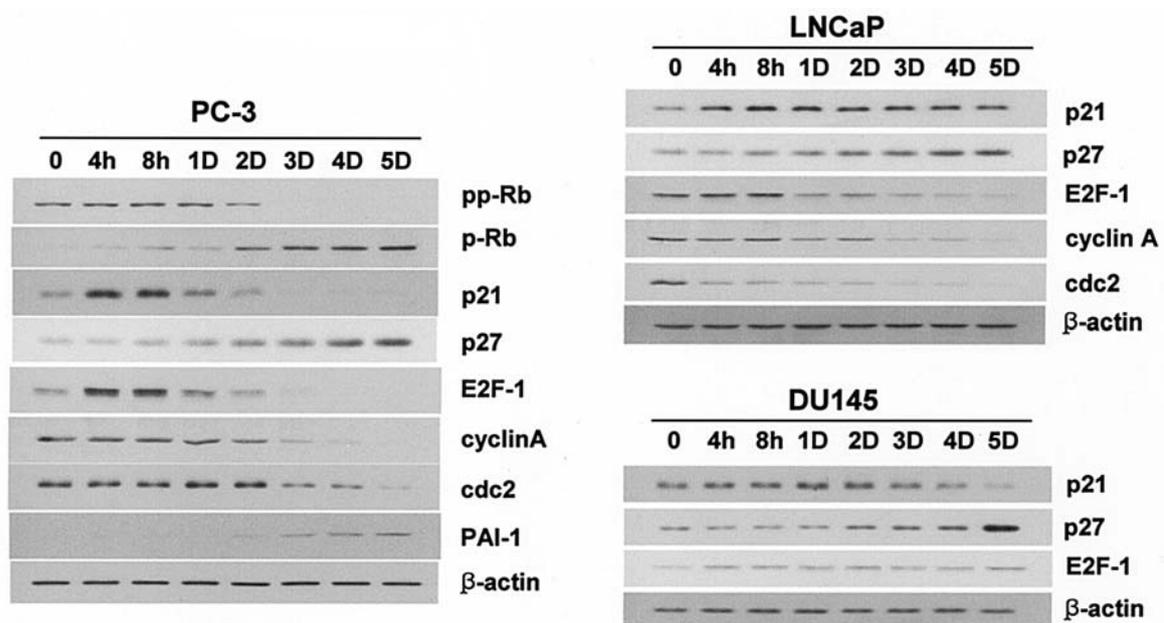


Figure 2. DNA-damage-induced senescence induces the reduction of E2F target gene expression. PC-3, DU145 and LNCaP cells were seeded at a concentration of 2×10^5 cells/ml prior to doxorubicin treatment, then harvested at the indicated intervals during exposure to 10 nM doxorubicin. The cells were lysed and 20 μ g of protein was loaded into each lane. Western blots were hybridized with the antibodies shown on the right. β -actin was used as a loading control.

2,5-diphenyl tetrazolium bromide (MTT) and with 5-bromo-4-chloro-3-indolyl β -D-galactopyranoside were purchased from Sigma (St. Louis, MO). Antibodies to E2F-1, cyclin A, cdc2, β -actin and E2Fs siRNA were obtained from Santa Cruz Biotechnology (Santa Cruz, CA); siRNA Rb kit was obtained from Cell Signaling Technologies (Beverly, MA). Enhanced chemiluminescence reagents (ECL) were from Amersham (Arlington Heights, IL). Immobilon-P transfer membrane for Western blot was purchased from Millipore

(Bedford, MA). The full-length E2F-1 open reading frame was cloned from normal fibroblast mRNA by reverse transcription-PCR for cloning into pCMVtaq4C (Invitrogen).

Cell culture and DNA transfection. Human prostate carcinoma DU145, LNCaP and PC3 cells were grown in RPMI-1640 supplemented with 10% (v/v) heat-inactivated FBS (Gibco Life Science, Grand Island, NY), 1% glutamine, and an antibiotic mixture added to a final concentration of 100 units/

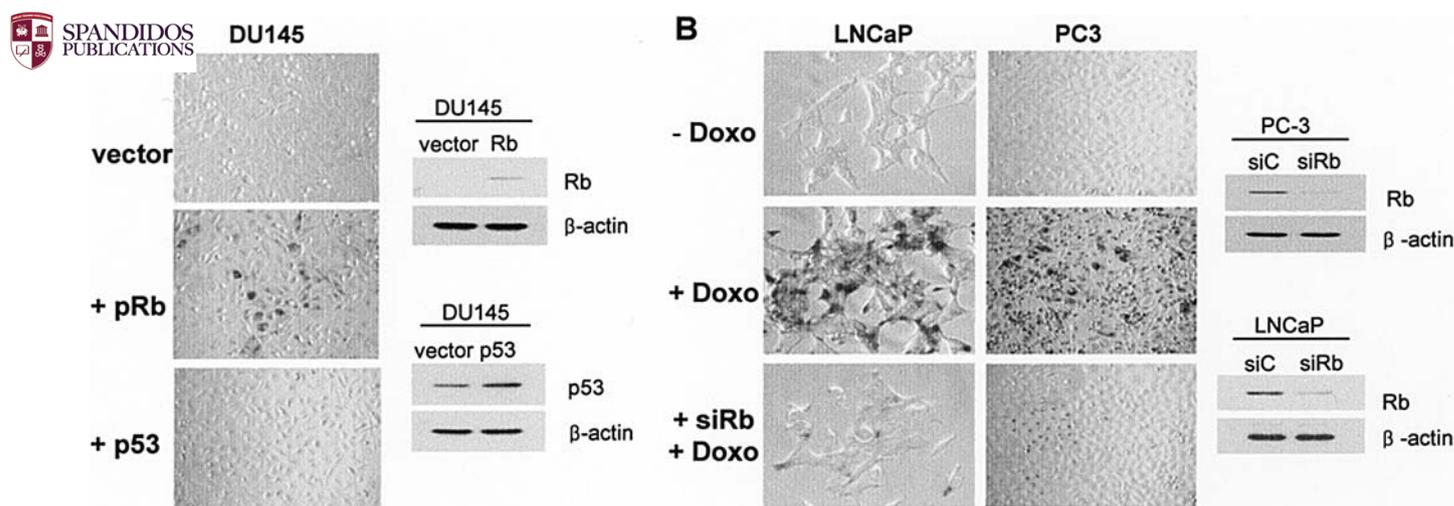


Figure 3. The effects of Rb or p53 on DNA damage-induced senescence. The human prostate carcinoma cell lines, PC-3, DU145 and LNCaP, were exposed to doxorubicin for 5 days, then stained for SA- β -gal. A, The p53- and pRb-mutant DU145 cells failed to stain positively for SA- β -gal. Subsequent to the introduction of Rb or p53 into DU145, the cells were exposed to doxorubicin for a further 5 days, and then stained positively for SA- β -gal in Rb-transfected DU145, demonstrating that pRb is a prerequisite for DNA damage-induced senescence. B, Inhibition of doxorubicin-induced senescence via Rb modulation. The siRNA-mediated knockdown of Rb eliminated senescence in both PC-3 (p53-null) and LNCaP (p53-wild). SiC, a non-targeted negative control duplex designed to monitor transfection efficiency using microscopy and to monitor siRNA specificity.

ml penicillin and 100 μ g/ml streptomycin. Cells were maintained in a humidified atmosphere containing 5% CO₂ at 37°C. Cells were transfected using the Amaxa electroporation system according to the supplier's protocol (kit T, program A-23). The transfection efficiency was >80% as assessed by co-transfection with DNA expressing GFP.

Western blot analysis. Western blots were performed as described previously. Briefly, cells were lysed in lysis buffer containing protease inhibitors [20 mM HEPES (pH 7.4), 150 mM NaCl, 1 mM MgCl₂, 1 mM EDTA, 2 mM EGTA, 10% glycerol, 1% Triton X-100, 1 μ g/ml leupeptin, 1 μ g/ml aprotinin, 2 mM Na₂VO₄ and 5 mM NaF]. Equal amounts (20 μ g) of cell lysates were resolved by 12% SDS-PAGE and subjected to Western blot analysis using the enhanced chemiluminescence system (Amersham Corp., Arlington Heights, IL). Antibodies used were Rb, underphosphorylated Rb (PharMingen, San Diego, CA), E2F-1, p16, p21, p27, Cdk5 (Santa Cruz, CA) at dilution of 1:1000, and β -actin (Sigma) for loading control.

Reverse transcription-PCR analysis of senescence marker. Expression of senescence marker was evaluated by reverse transcription-PCR. Total RNA was isolated using the RNeasy mini kit (Qiagen, Hilden, Germany) and treated with DNase I (Qiagen). The primer sequences designated from the coding region of the human PAI-1 cDNA are as follows: 5'-TGC TGG TGA ATG CCC TCT ACT-3' (sense) and 5'-CGG TCA TTC CCA GGT TCT CTA-3' (antisense); and human osteonectin cDNA are as follows: 5'-GGC CTG GAT CTT CTT TCT CCT T-3' (sense) and 5'-AAG AAG TGG CAG GAA GAG TCG AA-3' (antisense). The PCR conditions were as follows: 25 cycles of 95°C for 30 sec, 60°C for 30 sec, and 72°C for 30 sec, followed by a final incubation at 72°C for 10 min. The amplified product was analyzed by electrophoresis on an agarose gel and staining with ethidium bromide.

Senescence-associated β -Galactosidase (SA- β -Gal) and SAHF analysis. Cells were seeded into 6-well plates in RPMI-1640 culture medium. After 24 h, drugs were added, and the cells were incubated for 5 days. For SA- β -Gal staining, cells were fixed for 10 min in 2% formaldehyde and 0.2% glutaraldehyde, washed, and incubated at 37°C with 5-bromo-4-chloro-3-indolyl β -D-galactopyranoside (1 mg/ml), dissolved in a solution containing 40 mM citric acid (pH 6.5), 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 150 mM NaCl, and 2 mM MgCl₂. After a 24 h incubation, photographs were taken with a phase microscope. Cells were examined for SAHF analysis as described previously (17).

Results

Senescence is differentially induced in human cancer cells. Human cancer cells were compelled to senesce via treatment with the chemotherapeutic agent doxorubicin. After 5 days of exposure to doxorubicin, the features of cellular senescence including the activity of senescence-associated SA- β -galactosidase (SA- β -gal) were displayed in the PC-3 and LNCaP, but not in the DU145, which were mutated with regard to Rb, p53, and p16 (Fig. 1). In senescing human prostate cancer cells, underphosphorylated Rb, p21, p27, and senescence-specific genes such as PAI-1 or osteonectin were induced (Figs. 1 and 2). The levels of expression of hyper-phosphorylated Rb, E2F1, cdk and E2F target genes, including cyclin A and cdc2, were reduced within 5 days of drug exposure.

Rb or p53 is dispensable for the induction of cancer cell senescence. In the Rb-deficient, p53-mutated DU145 cells, forced expression of Rb induced senescence, but that was not the case with p53 (Fig. 3A). However, we were unable to dismiss the possibility that the introduced p53 may not have been active in the presence of the endogenous mutant p53 in DU145. The introduction of siRb prevented the

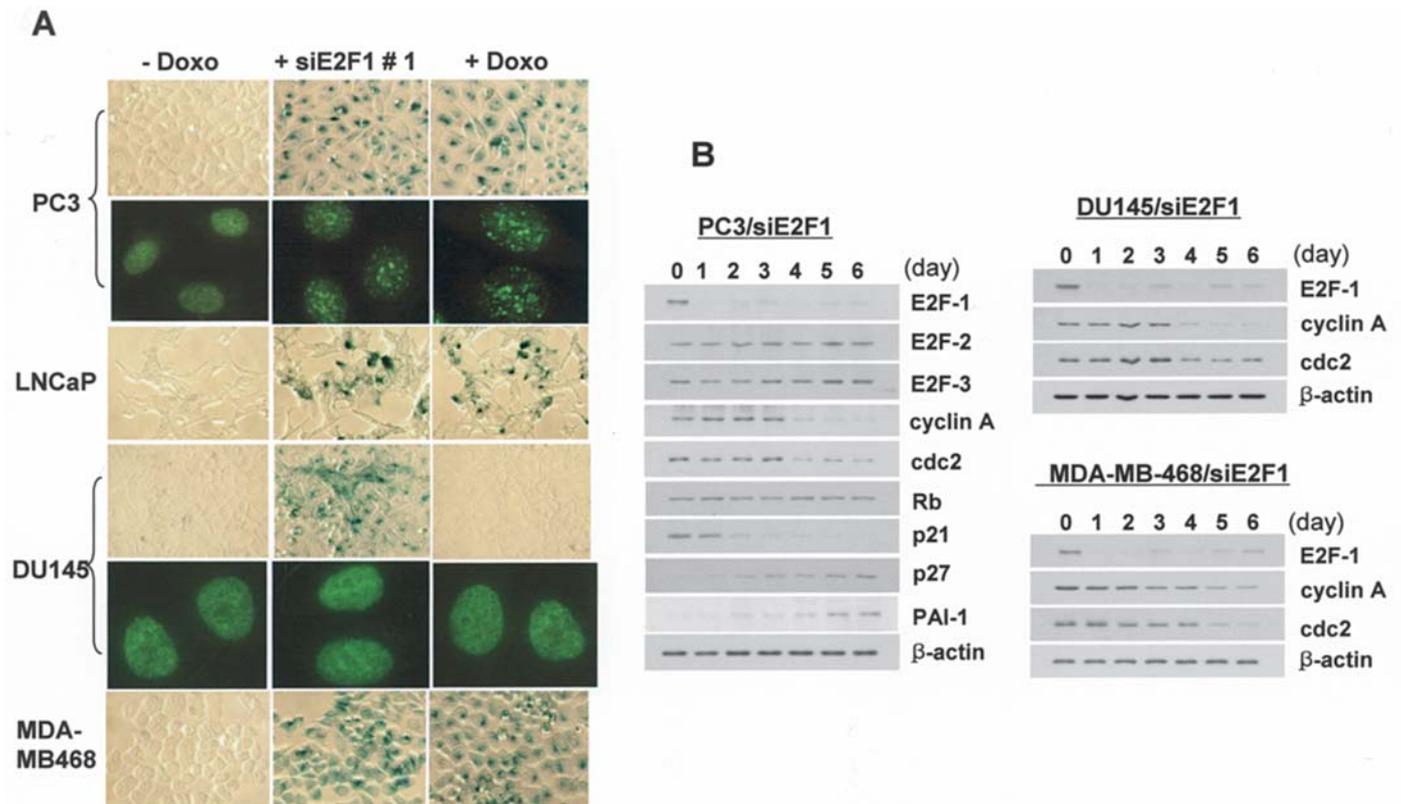


Figure 4. The siRNA-mediated knockdown of E2F1 induces senescence. PC-3, LNCaP, DU145 and MDA-MB-468 cells (2×10^6 cells) were exposed to doxorubicin or transfected with siE2F1 (100 nM) in Amaxa nucleofector solution, and were then subjected to SA- β -gal staining on day 5. Immunofluorescent images of methylation on lysine 9 (K9M) in the PC3 and DU145 cells are shown under the images of SA- β -gal staining. The cells were harvested at regular intervals, and were subjected to Western blot analysis for the expression of cell cycle or senescence-associated genes, as indicated on the right.

doxorubicin-induced accumulation of SA- β -gal in the PC3 and LNCaP cells under the same conditions (Fig. 3B). However, in MDA-MB-468, which is also Rb-/p53-, the results were quite different from those of DU145 with regard to SA-gal activity and gene expression (Fig. 4), whereas Rb may contribute to the mechanics of senescence, it does not appear to be an essential factor.

The siE2F1-induced senescence-phenotype is independent of Rb and p53. In order to understand the molecular basis for the apparent dissimilarity manifested by DU145 and MDA-MB-468, we analyzed E2F1. Western blot analysis confirmed that the cells into which siE2F1 had been introduced harbored substantially less E2F1 than did the control cells, but that E2F2 and E2F3 levels remained unaffected (Fig. 4). Control siRNA, which is a non-targeted negative control duplex, was designed to monitor transfection efficiency using microscopy and to monitor siRNA specificity. However, control siRNA did not induce senescence in any cell lines. Also, the levels of expression of the E2F target gene were reduced, indicating that siE2F1 mediated the functional inactivation of E2F (Fig. 4). We observed senescence-associated heterochromatic foci (SAHF) (17) in PC3, but not in Rb mutated DU145 cells (Fig. 4). The control cells continued to proliferate, and exhibited no senescence-associated changes. Our findings suggest that siE2F1 results in a senescence-reminiscent phenotype in human cancer cells. Thus, the depletion of E2F1 induced a senescence-like phenotype, as was evidenced by

stable growth arrest, morphology, and SA- β -gal activity in the Rb mutant DU145 and Rb-null MDA-MB-468 cells.

E2F1 inhibits the induction of senescence-like phenotype in cancer cells. Next, we attempted to determine whether the expression of E2F1 could prevent senescence in cancer cells. In our experiments, the E2F1-expressing cells were observed to escape DNA damage-induced accumulation of SA- β -gal (Fig. 5A). We also investigated whether forced expression of E2F-1 could prevent senescence-associated inhibition of cell proliferation in PC-3 cells. Ectopically overexpressed E2F-1 conferred a survival advantage on senescence-induced cells (Fig. 5B). Furthermore, FACS analysis of E2F-1-transfected cells demonstrated that senescence-induced cell cycle arrest was relieved by E2F-1 expression (Fig. 5C). Therefore, senescence, as evidenced by changes to the nuclear architecture and SA- β -gal, is clearly a consequence of the depletion of E2F1.

Discussion

The accumulation of senescence *in vivo* might be attributable to damaged or oncogene-stimulated cells (32,33). Here, we describe a negative role of E2F1 with regard to senescence in human cancer cells. Our results indicate that cancer cells respond to E2F1 depletion via the stable arrest of cell proliferation, which is quite similar to the properties observed in cellular senescence. This is not surprising, since E2F1 also

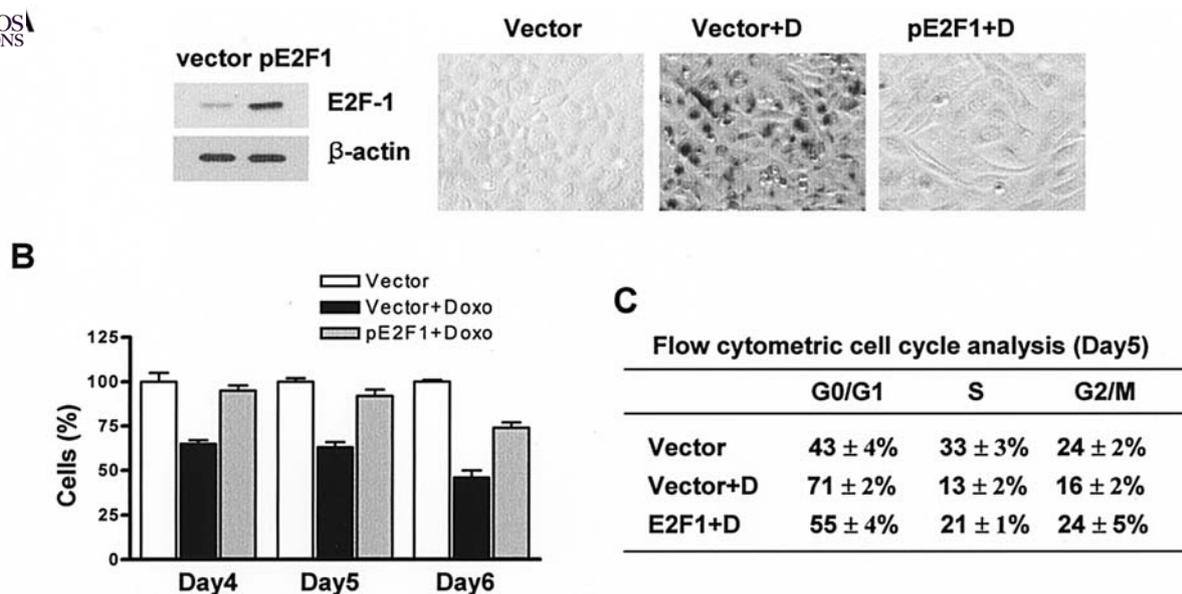


Figure 5. The prevention of doxorubicin-induced senescence via E2F1 overexpression. A, E2F1 expression vector (2 μ g) or control vector (2 μ g) was transfected into 2×10^6 of PC-3 cells in Amaxa nucleofector T solution, then plated onto cell culture dishes. The cells were then treated with 10 nM doxorubicin for 5 days, and the cells were assessed for SA- β -gal activity. B, Cell growth detailing the effect of E2F1 expression on the proliferation of PC-3 cells. E2F1- or vector-transfected cells were treated with 10 nM doxorubicin (D) on the following day and the cells were counted at day 4, 5 and 6. Cell growth was decreased by doxorubicin but partially recovered by E2F1 expression. C, Effects of E2F1 expression on the cell cycle of senescence-induced PC-3 cells. E2F1-transfected with vector-transfected cells were treated with doxorubicin (10 nM) for 5 days. Cells were fixed with 70% ethanol and incubated with RNase A and the DNA-intercalating dye propidium iodide. FACS analysis of E2F-1-transfected cells demonstrated that doxorubicin-induced cell cycle arrest was relieved by E2F-1 expression. Results of a representative experiment from three independent experiments are presented, and the means and standard deviations are given in the table.

induces growth stimulators. These findings may help to explain the apparently contradictory activities of E2F1 and the senescence-like phenotypes frequently observed in malignant tissues after the application of E2F1-suppressive chemotherapy.

The senescence response elicited by the depletion of E2F1 was determined to occur independently of the genetic background of the cell. Cells with compromised Rb or p53 senesced successfully in response to the depletion of E2F1. Furthermore, the ectopic-expression of E2F1 was shown to inhibit the induction of senescence. This result complements the published data which indicate that E2F1 stimulates DNA synthesis in quiescent immortal cells (20,23,27,28). Considering that the mitotic stimuli-induced E2F1 may inhibit or prevent senescence, and that the senescence-like growth arrest suppresses neoplastic transformation, we can conclude that malignant progression might also be affected by the modulation of E2F1. We have yet to fully understand the manner in which E2F1 depletion modulates senescence, and the manner in which senescence can be prohibited as the result of the expression of E2F1. Regardless of the underlying mechanism, our results suggest that the reduction of cyclinA and cdc2 is also a characteristic of senescent cancer cells. However, we do not know whether this is the result of Rb or p53, as these genes did not correlate sufficiently with the induction of senescence. Additionally, even considering the dramatic increase observed in the levels of p27, senescence was not induced in the DU145 cells, suggesting that the induction of p27 is not critical either, with regard to the induction of senescence.

Our results raise several important questions with regard to the manner in which cells can respond to senescence-inducing

stimuli, as well as the role of E2F1 in these responses. Little is currently known regarding the extent to which cells express E2F1 to undergo senescence. Moreover, human cancer cells are known to differ in their propensity for E2F1 expression. One possibility is that continuous DNA damage puts cells at risk for inaccurate repair, which can cause cell cycle arrest and E2F1 down-regulation, culminating in senescence.

Cellular senescence is a stable form of cell cycle arrest, which limits the proliferation of damaged cells. Narita *et al* described senescence-associated heterochromatic foci (SAHF) as distinct senescence-specific chromatin structural formations, which involve the recruitment of heterochromatin proteins and the Rb to E2F-responsive promoters, which are associated with the stable repression of E2F target genes (17). Our data indicate that the down-regulation of E2F1 ensures the arrest of senescence. Notably, E2F1 depletion-induced cancer cell senescence occurs independently of the integrity of both Rb and p53. The formation of SAHF was found to be strictly dependent on the presence of Rb, but senescence was clearly observed in the SAHF-negative DU145 cells.

In senescence, Rb and p53 were not indispensable, and the depletion of E2F1 was determined to assume the role in cancer-cell senescence that was normally played by functional Rb or p53. As cancer cells are largely deficient of both functional Rb and p53, it would appear to be more feasible to deplete E2F1 than to recover functional Rb or p53 in the treatment of cancer. On the basis of our results, we conclude that E2F1 may be required not only for the progression of the cell cycle, but may also constitute a prerequisite for the suppression of senescence, thereby contributing to the modulation of carcinogenesis.

Acknowledgements

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