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14. ABSTRACT Mammalian cells can respond to stress or damage by undergoing a permanent cell cycle arrest termed cellular senescence. The senescence response suppresses the development of cancer, but the altered cellular functions that accompany this response may contribute to aging. Our study addressed whether and how mouse senescent stroma contributes to breast cancer and what role senescent mouse fibroblasts have in this process. We established for the first time an adequate and reliable in vitro mouse model mimicking human senescence, with extensive similarities. 3% oxygen culture condition combined with X-irradiation is the prerequisite to imitate human fibroblast senescence. Under these conditions, human and mouse senescent stromal cells disrupt normal mammary epithelial differentiation, and promote hyper-proliferation of pre-neoplastic mammary epithelial cells. Our findings suggest that senescent fibroblasts can disrupt the stromal-epithelial interactions, ultimately promoting breast cancer with age. By establishing such a mouse model, we hope to improve the scientific possibilities exploring the effect of aging on breast cancer.					
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Final Report

Agging, breast cancer and the mouse model

Introduction

Background

Studies of human diseases widely use the laboratory mouse as a model organism. Mouse models have provided the basis for the establishment of fundamental concepts in the understanding of breast cancer development and progression. Genetically engineered mice have shown that molecular lesions involved in human breast cancers also induce mammary cancers in the mouse and that these tumors have strikingly similar histology. Nonetheless, several limitations exist to this model system and significant differences exist in the structure, development and physiology of the mammary gland and even more extensive differences can be found in the tumorigenesis of the two species. Thus, our research was aimed at addressing the similarities and differences between mouse and human breast cancer biology, in order to develop mouse models that better mimic the biology of age-related human tumors.

In humans, the incidence of most cancers, including breast cancer, rises exponentially with age (ref. 1-3). It is believed that this increase results from a synergy between the accumulation of somatic mutations over a lifetime and age-dependent epigenetic factors. It is well established that the growth and differentiation of normal breast epithelial cells is highly dependent on the stromal microenvironment. The microenvironment strongly influences the development and progression of breast epithelial carcinogenesis, and alterations in the stroma can create a pro-carcinogenic milieu. One hypothesis is therefore that age-dependent changes in tissue environments may synergize with the time-dependent accumulation of somatic mutations in epithelial cells to produce cancer later in life. Recently, our group found that senescent human fibroblasts stimulate premalignant epithelial cells to proliferate in culture and nude mice, suggesting that cellular senescence might be one such epigenetic factor (ref. 3,4).

Senescence is a fundamental property of normal somatic cells. It limits division by imposing an irreversible block to cell-cycle progression. In human cells, replicative senescence occurs when the telomeres shorten to a critical length. Senescence can also be induced by DNA damage, including oxidative stress and X-irradiation, oncogene expression and supraphysiological mitogenic stimulation. Senescence irreversibly arrests the growth of cells at risk for neoplastic transformation. Cellular senescence also entails major phenotypic changes, among which are alterations in differentiated functions. Senescent cells also exist and accumulate with age *in vivo*. Taken together, these results suggest that senescent cells can alter the environment of the mammary epithelium late in life, when they accumulate, thereby creating a permissive environment for the expression of malignant phenotypes.

Mouse embryo fibroblasts (MEFs) are the most common model system for studying mouse replicative growth arrest in culture. Despite the widespread tendency to equate replicative senescence in human and mouse cells, many differences exist between the two systems. MEFs rapidly senesce in culture prior to a quick immortalization of the cell population, whereas, in human cells, replicative senescence only occurs after many more cell cycles and never exit from the senescent state. Mouse telomeres are significantly longer than human telomeres and do not experience much attrition before senescence, suggesting that the replicative growth arrest of mouse cells is telomere-independent. Thus

our aim was to extensively characterize the senescent phenotype of murine fibroblasts compared to human fibroblasts in the context of aging and tumor development.

Rational

The goal of this project was to understand whether and how mouse stroma contributes to breast cancer, and what role senescent mouse fibroblasts have in this process in order to model human age-related cancer in the mouse and learn from this model system. It was necessary to assess whether the mouse can be adequately used for studying the effects of cellular senescence on cancer development and progression. Determining the relevance and limitations of the mouse model to study the role of cellular senescence and aging in breast cancer necessitated identifying the commonalities and differences between the human and mouse senescence pathways, at the cellular and tissue-microenvironment level.

Body

We have addressed all the main objectives presented in the original proposal. We identified conditions to mimic adequately human senescence in culture, also allowing us to study breast cancer phenotypes in culture. We thus have succeeded in establishing a new mouse model for human aging, identifying mouse senescent fibroblasts as pro-carcinogenic effectors of the tissue microenvironment.

Senescence in human vs mouse fibroblasts

Many murine cells undergo only a few doublings in culture, despite long telomeres and expression of telomerase, before arresting transiently in a cellular state originally assumed to be a human-like senescence. We have shown that these cells most likely arrest because standard culture conditions cause severe oxidative stress [Appendix #1] (ref. 5). When cultured in physiological oxygen (3% oxygen culture, or normoxic conditions), murine fibroblasts can proliferate indefinitely and avoid replicative senescence altogether. They do so with intact p53 function. Importantly, MEFs which typically senesce after approximately 10 doublings in hyperoxic conditions suffer from a high level of DNA damage in 20% oxygen (standard atmospheric culture conditions, or hyperoxic conditions), and suffer more DNA damage than human cells grown in the same conditions. This indicates that human cells are more capable of withstanding oxidative stress either through an increased ability to neutralize ROS or to monitor and repair DNA. The senescence response of MEFs to oxygen-induced DNA damage is mediated through p53, as p53-null MEFs do not senesce under hyperoxic conditions. Crucially, in contrast to mouse fibroblasts that spontaneously arrest growth in 20% oxygen, mouse fibroblasts cultured in 3% oxygen and subsequently X-irradiated behave similarly to human fibroblasts made senescent by replicative exhaustion or X-irradiation [Appendices #1, #4, #6]. Further biochemical analysis of the mouse senescence response in culture has been characterized. In particular, the c-fos response to serum observed in human senescent fibroblasts can be recapitulated in mouse fibroblasts senesced in normoxic conditions, but not in hyperoxic cultures. Additionally, quantitative RT-PCR for genes known to be up-regulated in human senescent fibroblasts (such as MMP-1, MMP-3 or PAI-1) indicate a conserved transcriptome in mouse cells senesced at 3% but not at 20% oxygen. Thus these similarities led us to believe it was possible to induce a human-like senescence in mouse fibroblasts when cultured in normoxic conditions only.

Senescent mouse fibroblasts disrupt normal epithelial cell differentiation

Our ultimate goal is to establish a mouse model for breast cancer and aging, whereby the senescent stroma alters the tissue microenvironment and disrupt epithelial cell normal physiology. Thus, comparing the effects of mouse and human senescent fibroblasts (“aged-fibroblasts”) on the surrounding epithelial cells was the critical next step.

A 3D mammary epithelial cell (MEC) co-culture assay was used to study branching morphogenesis, a process that requires factors produced by fibroblasts. This assay measures the contribution of surrounding stromal cells to the induction and maintenance of ductal branching of MECs in 3D. MECs were isolated as multicellular organoids from virgin mouse mammary glands. We culture the organoids in a 3D collagen I gel containing embedded fibroblasts, mimicking the mammary stroma. Alternatively, to test the effects of factors secreted by fibroblasts on MEC morphology, conditioned medium from fibroblasts is added to MEC 3D cultures. Under both conditions, within 7-10 days, the epithelial cells invade the stroma to form branched ductal structures similar to those in the developing gland. The size, number, type and length of branches are quantified by an image analysis program (developed in-house). The 3D branching morphogenesis assay showed that senescent fibroblasts stimulated excessive branching, resulting in many more primary and secondary branches and even tertiary branches. This was found to be due to over-expression of MMP3 [Appendix #2, #4] (ref. 6). In addition to these changes in branching morphogenesis, there was an concomitant stimulation of epithelial cell proliferation. Conditioned medium could substitute for senescent fibroblasts, emphasizing the importance of the SSP on neighboring cells. In another assay (3D functional alveolar differentiation assay), senescent, but not presenescent, mouse or human fibroblasts substantially increased the number of large misshapen (and less organized) alveoli formed by mouse or human MECs. Senescent fibroblasts decreased the amount of b-casein produced by the mouse MECs.

These data support the idea that senescent cells can contribute to age-related pathology, including cancer, and describe a new property of senescent stromal cells – the ability to disrupt epithelial differentiation. We conclude that senescent mouse fibroblasts disrupt mammary epithelial cell functional differentiation and branching morphogenesis. MMP-3 is the major secreted factor responsible for the effects of senescent fibroblasts on branching morphogenesis. This molecular phenotype is conserved between mouse fibroblasts senesced by X-irradiation in 3% oxygen and human senescent fibroblasts.

Senescent mouse fibroblasts promote growth and transformation of premalignant and malignant epithelial cells

Co-culture assay. Two dimensional (2D) co-culture assays were used to measure growth stimulation by cell-cell contact and/or paracrine factors. A small number of epithelial cells are seeded on a “feeder layer” of presenescent or senescent fibroblasts previously shifted to serum-free medium for 2-3 days to arrest growth the growth of presenescent cells (senescent cells are already arrested). Epithelial colonies are discernible 7-10 days later. Epithelial cell growth is quantified using an imaging program and either of two methods. 1) We express a green fluorescent protein (GFP) derivative in the epithelial cells, and measure green fluorescence. 2) We stain the co-cultures with the nuclear dye DAPI (diamidino-2-phenylindole) and count nuclei based on the relatively small size of epithelial nuclei compared to fibroblast nuclei. A variation to this assay is an indirect co-culture assay which measures growth stimulation by diffusible factors in 2D. A fibroblast lawn is placed on the bottom of a lower chamber in a two-chamber culture dish (Boyden chamber). The chambers are separated by a porous membrane that allows passage of diffusible molecules, but not cells. We seed epithelial cells on the upper side of the membrane. After 5-10 days in serum-free medium the number of epithelial cells in the upper chamber are counted. As an alternative, conditioned media from fibroblasts is collected, filtered and added to the epithelial cells in a separate culture dish and the proliferative index measured.

The 2D co-culture assay showed that senescent human fibroblasts significantly stimulated the proliferation of premalignant and malignant epithelial cells when compared to presenescent fibroblasts. Mouse and human mammary epithelial cells were used: a premalignant cell line Scp2 (mouse mammary epithelial cells), a variant of the premalignant cell line EpH4 (EpH4v, mouse mammary epithelial cells). Adult and embryonic human fibroblast were used (primary human breast fibroblasts and two embryonic lung fibroblast lines). Co-cultures were assayed either at 20% or at 3% oxygen. Hyperoxic and normoxic

conditions showed senescent-induced hyperproliferation of epithelial cells [Appendix #3]. Soluble factors secreted by fibroblasts in normoxic conditions are responsible, at least in part, for MEC hyperproliferation. Soluble factors from senescent fibroblasts also induce scattering of epithelial cell colonies. This induced phenomena resembles epithelial cell de-differentiation and is reminiscent of a possible epithelial-mesenchymal transition (EMT). Induction of growth and scattering is one hallmark of cancer, indicative of a pro-tumorigenic activity induced by soluble factors.

Similarly, we attempted to reproduce the effects of senescent human fibroblasts on premalignant epithelial cells using “replicatively senescent” (20% oxygen) mouse embryo fibroblasts (MEFs) [Appendix #3]. Replicatively senescent mouse embryo fibroblasts in hyperoxic culture conditions (MEFs, 20%) did not stimulate the growth of premalignant epithelial cells. In contrast, MEFs cultured in 3% oxygen and then induced to senesce by IR, induced epithelial proliferation, essentially behaving like human cells in 20% oxygen. These findings support our previous conclusions that the replicative arrest of mouse cells in 20% oxygen is due to severe oxidative damage, which has now been shown to produce a phenotype distinct from that of senescence induced in physiological oxygen. They are consistent with our finding that MEFs accumulate many more oxidative DNA lesions and chromosome breaks in 20% oxygen than similarly cultured human cells. For this reason, all experiments using mouse cells as a model of human senescence are now performed in a 3% oxygen atmosphere.

All together, these studies suggest that stromal cells from human and mouse senesced in normoxic conditions only have a conserved phenotype. Human and mouse senescent fibroblasts can create a pro-cancer microenvironment for premalignant and malignant epithelial cells within the vicinity of the mammary gland. Senescence, once reached, may have some preserved biological roles triggered by preserved downstream molecular properties, mainly influencing neighboring cells. These data also suggest that, as senescent cells accumulate *in-vivo*, in mice or humans, they may promote cancer in aged organisms. Finally, in the case of mouse cell culture, normoxic conditions are necessary to recapitulate a “true” senescent phenotype.

Key Research Accomplishments

Manuscripts:

Parrinello S et al. (2003) Oxygen sensitivity severely limits the replicative life span of murine cells. *Nature Cell Biol.* 5: 741-747.

Parrinello S et al. (2005) Stromal-epithelial interactions in aging and cancer: senescent fibroblasts alter epithelial cell differentiation. *JCS* 118: 485-496.

Coppe JP et al (*manuscript in preparation*) The conserved pro-inflammatory secretory phenotype of senescent human and mouse fibroblasts.

Meeting Abstracts:

Coppe JP et al, Keystone Meeting (Senescence and Apoptosis), 2005, Inducing a human-like senescent phenotype in mouse fibroblasts

Krtolica A et al, Keystone Meeting (Senescence and Apoptosis), 2005, Senescent stromal cells alter mammary gland differentiation

Coppe JP et al, US Army MR&MC (Era of Hope - Breast Cancer Program), 2005, Inducing a human-like senescent phenotype in mouse fibroblasts promotes mammary epithelial cell proliferation and tumorigenesis: a mouse model for human aging.

Reportable Outcomes

Manuscripts:

See appendices for reprints of the listed Key Research Accomplishment

Parrinello S et al. (2003) Oxygen sensitivity severely limits the replicative life span of murine cells. *Nature Cell Biol.* 5: 741-747.

Parrinello S et al. (2005) Stromal-epithelial interactions in aging and cancer: senescent fibroblasts alter epithelial cell differentiation. *JCS* 118: 485-496.

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See appendices for reprints of the listed Key Research Accomplishment

Coppe JP et al, Keystone Meeting (Senescence and Apoptosis), 2005, Inducing a human-like senescent phenotype in mouse fibroblasts

Krtolica A et al, Keystone Meeting (Senescence and Apoptosis), 2005, Senescent stromal cells alter mammary gland differentiation

Coppe JP et al, US Army MR&MC (Era of Hope - Breast Cancer Research Program), 2005, Inducing a human-like senescent phenotype in mouse fibroblasts promotes mammary epithelial cell proliferation and tumorigenesis: a mouse model for human aging.

Meeting Presentation / Invited Speaker:

Coppe JP, US Army MR&MC (Era of Hope - Breast Cancer Research Program), 2005, “Cellular Senescence, Aging, and Breast Cancer”.

Conclusions

Mammalian cells can respond to stress or damage by undergoing a permanent cell cycle arrest termed cellular senescence. The senescence response suppresses the development of cancer, but the altered cellular functions that accompany this response may contribute to aging. The goal of our study was to understand whether and how mouse stroma contributes to breast cancer and what role senescent mouse fibroblasts have in this process. To do so, we attempted to determine the relevance and limitations of the mouse model for studying the role of cellular senescence and aging in breast cancer, in comparison to human senescence. We have been successful in establishing appropriate *in vitro* conditions for mouse fibroblasts to mimic the physiological effects of human stromal aging. We defined at the molecular level how fibroblasts can affect their microenvironment, in particular in models of human and mouse mammary gland carcinogenesis, where senescent fibroblast disturb the stromal-epithelial interactions. Our data provide a basis for creating an *in vivo* mouse model of human cellular senescence, and studying effects of senescent stromal cells on aging and cancer *in vivo*. Once such a model is established, it will have much improved applications for exploring the effects of aging on breast cancer and for developing new treatments aimed to counteract senescent changes in the microenvironment.

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6. Parrinello S, Coppe JP, Krtolica A, Campisi J. (2005) Stromal-epithelial interactions in aging and cancer: senescent fibroblasts alter epithelial cell differentiation. JCS 118: 485-496.

Appendices

Appendix #1: Publication (pdf.file): Parrinello S et al, 2003, Nature Cell Biology

Appendix #2: Publication (pdf.file): Parrinello S et al, 2005, J. Cell Science

Appendix #3: Figure 1 (pdf.file)

Appendix #4: Abstract: Coppe JP et al, Keystone Meeting (Senescence and Apoptosis), 2005

Appendix #5: Abstract: Krtolica A et al, Keystone Meeting (Senescence and Apoptosis), 2005

Appendix #6: Abstract: Coppe JP et al, US Army MR&MC (Era of Hope - BCRP), 2005

Oxygen sensitivity severely limits the replicative lifespan of murine fibroblasts

Simona Parrinello¹, Enrique Samper², Ana Krtolica¹, Joshua Goldstein¹, Simon Melov² and Judith Campisi^{1,2}

Most mammalian cells do not divide indefinitely, owing to a process termed replicative senescence. In human cells, replicative senescence is caused by telomere shortening, but murine cells senesce despite having long stable telomeres¹. Here, we show that the phenotypes of senescent human fibroblasts and mouse embryonic fibroblasts (MEFs) differ under standard culture conditions, which include 20% oxygen. MEFs did not senesce in physiological (3%) oxygen levels, but underwent a spontaneous event that allowed indefinite proliferation in 20% oxygen. The proliferation and cytogenetic profiles of DNA repair-deficient MEFs suggested that DNA damage limits MEF proliferation in 20% oxygen. Indeed, MEFs accumulated more DNA damage in 20% oxygen than 3% oxygen, and more damage than human fibroblasts in 20% oxygen. Our results identify oxygen sensitivity as a critical difference between mouse and human cells, explaining their proliferative differences in culture, and possibly their different rates of cancer and ageing.

Replicative senescence limits the proliferation of many cell types in culture and *in vivo*². Human cells senesce replicatively largely because telomeres — DNA structures that cap chromosome ends — shorten and malfunction when replicated in the absence of telomerase, which most human cells do not express¹. Senescent cells adopt a distinctive morphology and pattern of gene expression^{2,3}, a phenotype also induced by telomere-independent events^{2,4} such as DNA damage and activation of certain oncogenes. The senescence response is thought to suppress cancer².

MEFs are used widely in the study of replicative senescence, despite notable differences between human and rodent cells^{1,2}. MEFs spontaneously overcome replicative senescence (immortalization)^{5,6}, whereas human fibroblasts rarely do so. Moreover, MEF senescence relies predominantly on the p19^{ARF}/p53 tumour suppressor pathway, whereas human fibroblasts require loss of both p53 and retinoblastoma (Rb) tumour suppressor functions for immortalization^{2,7}. Furthermore, MEFs senesce after many fewer population doublings than human fibroblasts, despite having longer telomeres and constitutively expressing telomerase^{1,2}. These differences suggested that MEFs senesce as a result of culture stress, the nature of which is unknown⁸.

Here, we compared the phenotypes of senescent mouse and human fibroblasts. We cultured MEFs from C57Bl/6 (or FVB; data not shown)

mice under standard conditions, which include atmospheric (20%) oxygen (Fig. 1a). As expected⁵, the cells grew well for approximately 1 week (2–3 population doublings) before proliferation began to decline. After 4–5 weeks (8–10 population doublings), the cultures senesced (arrow), showing no change in cell number for more than 1 week and a senescent morphology. Proliferation eventually resumed, owing to outgrowth of immortal variants⁵.

We monitored the capacity for DNA synthesis at each passage by labelling cells with ³H-thymidine for 3 days and counting radiolabelled nuclei. As reported⁹, the percentage of labelled nuclei declined progressively (Fig. 1b). However, it never fell below 25%, despite no increase in cell number at senescence (Figs 1a, b). Thus, we conclude that some senescent MEFs synthesized DNA, possibly reflecting endoreduplication or unscheduled DNA synthesis. In contrast, senescent human fibroblasts completely cease DNA synthesis when analysed with the same assay^{2,10}. In addition, replicatively senescent human fibroblasts failed to express c-Fos when stimulated with serum¹⁰, but senescent MEFs fully retained c-Fos inducibility (Fig. 1c). Thus, the phenotypes of senescent mouse and human fibroblasts are different.

Senescent MEFs resembled human fibroblasts induced to senesce by treatment with hydrogen peroxide, a strong oxidant¹¹. As previously reported¹¹, hydrogen-peroxide-treated human fibroblasts arrested growth, developed a senescent morphology and expressed senescence-associated β -galactosidase¹¹. However, despite no increase in cell number, the percentage of labelled nuclei was 30–40% and c-Fos remained inducible (Fig. 1d). Retention of c-Fos inducibility was specific to hydrogen-peroxide-induced senescence. Cells induced to senesce by treatment with the DNA-damaging agent bleomycin¹² or through overexpression of p14^{ARF} (ref. 13) lost serum-inducible c-Fos expression (Fig. 1e). These findings suggest that MEFs senesce as a result of oxidative stress.

To test this idea, we cultured MEFs in 3% oxygen. We found that they grew faster (doubling times 25 h in 3% oxygen versus 38 h in 20% oxygen) and reached higher saturation densities ($4.9 \times 10^3 \text{ cm}^{-2}$ in 3% oxygen versus $2.4 \times 10^3 \text{ cm}^{-2}$ in 20% oxygen) in 3% oxygen (Fig. 2a). Strikingly, cells showed no signs of senescence (Fig. 2a). Rather, the cultures grew continually, reaching in excess of 60 population doublings. Similar results were obtained using MEFs from nine C57Bl/6 embryos, four FVB (data not shown) and eight C57Bl/6/129 embryos (see Figs 3b and Supplementary Information, Fig. S2d). Although it is possible that some cells senesce in 3% oxygen and that

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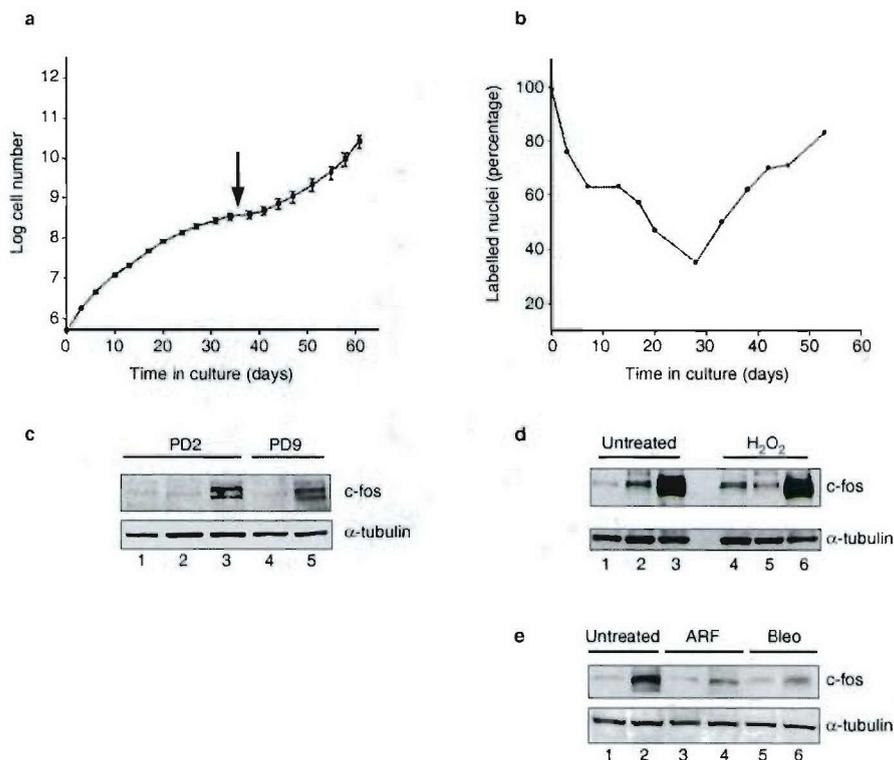


Figure 1 Senescent MEFs resemble oxidant-treated human fibroblasts. (a) Replicative lifespan, senescence (arrow) and spontaneous immortalization (resumed increase in cell number) is shown for the average of three independent C57BI/6 MEF cultures (\pm s.d. indicated by error bars). (b) The percentage of labelled nuclei for a C57BI/6 MEF culture were calculated at each passage for 55 days. Three independent cultures gave similar results. (c) Either early passage (PD2) or senescent (PD9) C57BI/6 MEFs were analysed by western blotting for c-Fos and α -tubulin (control). Proteins (30 μ g) from proliferating (lane 1), serum-deprived (lanes 2, 4) or serum-stimulated (lanes 3, 5) were analysed. (d) 82-6 human fibroblasts were treated with 400 μ M hydrogen

peroxide for 2 h. After 7 days, control and treated cells were maintained in 10% FCS (lanes 1 and 4), serum-deprived (lanes 2 and 5) or serum-deprived and stimulated (lanes 3 and 6). Proteins were analysed by western blotting for c-Fos and α -tubulin. c-Fos induction was detected 7 and 14 days after treatment with 200, 400 or 550 μ M hydrogen peroxide. (e) Human fibroblasts were induced to senesce by infection with pLXSN-p14^{ARF} (ref. 13) or treatment with 20 μ g ml⁻¹ bleomycin for 2 h (ref. 12). After 7 days, control (untreated), infected (ARF) and treated (Bleo) cells were serum-deprived (lanes 1, 3 and 5) or serum-stimulated (lanes 2, 4 and 6) and analysed by western blotting for c-Fos and α -tubulin.

minor strain-specific differences exist (see, for example, Supplementary Information, Fig. S2d), in all cases MEFs grew with little evidence of replicative senescence in 3% oxygen.

MEFs cultured for many population doublings in 3% oxygen showed normal cell-cycle arrest after DNA damage. When we X-irradiated MEFs grown for 24 population doublings in 3% oxygen, the fraction of cells in S phase declined, similarly to their behaviour at early passage (Table 1). MEFs constitutively expressed telomerase in 3% oxygen (data not shown), as reported for MEFs in standard culture⁶. Thus, when cultured in physiologically low levels of oxygen¹⁴, MEFs behaved like human fibroblasts expressing telomerase: that is, replicative senescence did not occur, but the cells retained normal growth control^{1,2}.

To further characterize MEFs cultured in physiological levels of oxygen, we passaged MEFs for 8, 13 or 19 population doublings in 3% oxygen and then shifted them to 20% oxygen. PD8 and PD13 cultures underwent 5–6 additional doublings, after which they senesced (Fig. 2b). PD19 cultures slowed growth slightly, then proliferated similarly to controls maintained in 3% oxygen (Fig. 2b). Thus, MEFs cultured in 3% oxygen remained sensitive to 20% oxygen for 10–15 population doublings, but eventually accumulated 20% oxygen-unresponsive variants. This

finding suggests the occurrence of a mutagenic or adaptive event in 3% oxygen that allows MEFs to overcome the 20% oxygen-triggered arrest.

To examine this event, we measured expression of p19^{ARF} and p16, growth-inhibitory tumour suppressors that upregulate p53 and Rb activity. Western blot analysis showed that early passage 3% oxygen cultures expressed low levels of p16 and p19^{ARF} and that levels rose with increasing passage (Fig. 2c). Levels of p19^{ARF} were variable in later-passage 3% oxygen cultures, but remained inducible (see below). However, growth in 3% oxygen did not generally result in loss of p53 function (Fig. 2d–g and Table 1). After X irradiation, late-passage 3% oxygen cultures showed stabilization of p53 levels (Fig. 2d), induction of p21 (Fig. 2e) and reduced DNA synthesis (Table 1; percentage labelled nuclei not shown), which was p53-dependent. We expressed a dominant p53 inhibitor, GSE-22 (ref. 15), in early and later-passage 3% oxygen MEFs and confirmed that it increased p53 levels, as previously reported¹⁵ (see Supplementary Information, Fig. S1a). Control cultures underwent cell-cycle arrest after X irradiation, but GSE-expressing cultures did not. Additionally, ectopic p19^{ARF} expression reduced DNA synthesis (percentage labelled nuclei) in four independent 3% oxygen cultures, but not in two immortal cultures that arose

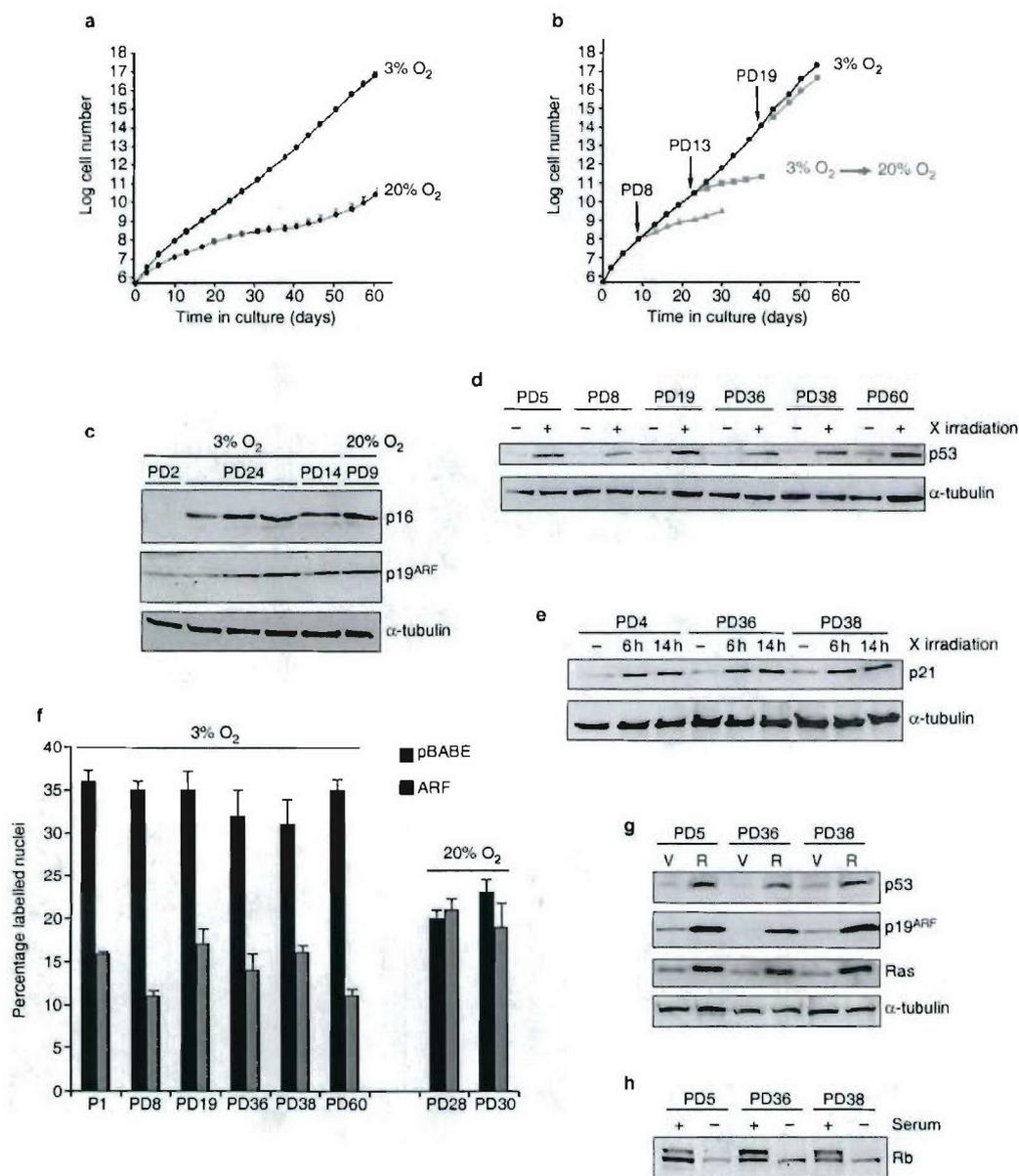


Figure 2 Low oxygen abolishes replicative senescence of MEFs. C57Bl/6 MEFs were used for all experiments. (a) MEFs were cultured in 20% or 3% oxygen, as indicated, and cell number was determined at each passage. The average and standard deviations of three independent cultures are shown. (b) MEFs cultured in 3% oxygen (black) were shifted at PD8, PD13 or PD19 to 20% oxygen (grey), or maintained in 3% oxygen. Cell number was determined at the indicated times. The average of two cultures is shown. (c) MEFs cultured in 3% or 20% oxygen were assayed for levels of p16, p19^{ARF} and α -tubulin (control) by western blotting. 3% oxygen cultures were analysed at early (PD2) and late (PD14 and PD24) passage. A senescent 20% oxygen culture (PD9) is shown for comparison. (d) Six 3% oxygen MEF cultures at the indicated population doublings were analysed for levels of p53 and α -tubulin by western blotting before (-) or 1 h after X irradiation (+; 4.5 Gy). (e) An early

passage (PD5) and two late-passage (PD36 and PD38) 3% oxygen MEF cultures were analysed for levels of p21 and α -tubulin before (-), 6 h and 14 h after X irradiation (4.5 Gy). (f) Six 3% oxygen MEF cultures at the indicated population doublings and two 20% oxygen immortal cultures were infected with control (black bars) or p19^{ARF}-expressing (grey bars) retroviruses. After 48 h, ³H-thymidine was added for 1 h and the S-phase fraction determined (percentage labelled nuclei). (g) An early passage (PD5) and two late-passage (PD36 and PD38) 3% oxygen MEF cultures were infected with insertless vector (V) or Ha-Ras^{V12}-expressing (R) retroviruses and assayed for p19^{ARF}, p53, Ras and α -tubulin by western blotting. (h) Exponentially growing (+) and serum-deprived (-) early passage (PD5) and late-passage (PD36 and PD38) 3% oxygen MEFs were assayed for levels of Rb by western blotting. Equal loading was confirmed by Ponceau S staining.

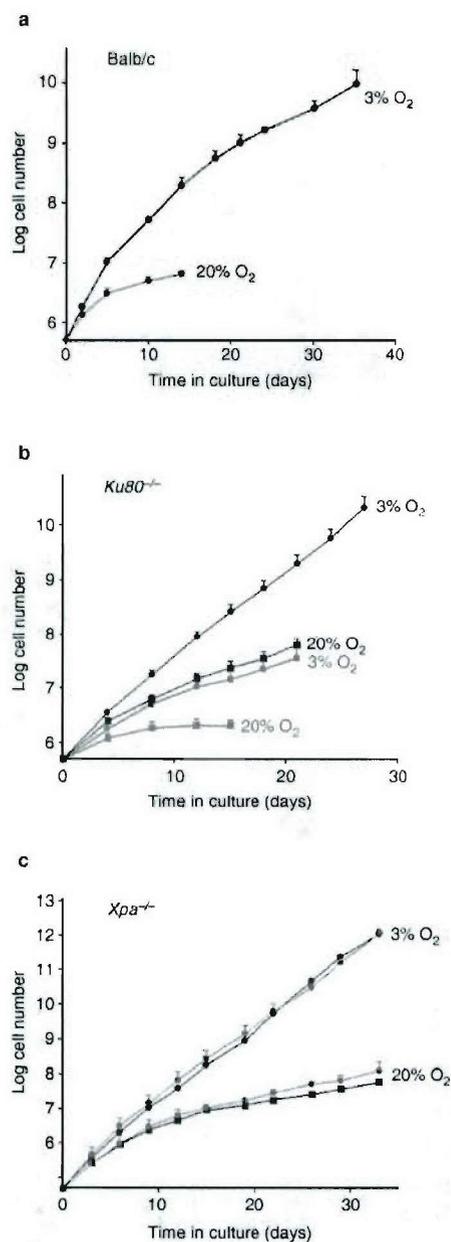


Figure 3 Growth of repair-deficient MEFs in 3% and 20% oxygen. (a) The replicative lifespans of three independent Balb/c MEF cultures maintained in 3% oxygen and two cultures maintained in 20% oxygen are shown. Error bars indicate the standard deviations. (b) Replicative lifespans and standard deviations of five *Ku80*^{-/-} (grey) and three wild-type littermate (black) MEF cultures in 3% (circles) and 20% (squares) oxygen were determined. (c) The replicative lifespans and standard deviations of two *Xpa*^{-/-} (grey) and two wild-type littermate (black) MEFs cultures grown in 3% (circles) and 20% (squares) oxygen are shown.

spontaneously in 20% oxygen (Fig. 2f). Furthermore, expression of oncogenic Ras, which induces p19^{ARF} and p53 in early passage 20% oxygen MEFs¹⁶, induced p19^{ARF} and p53 in both early and later passage 3% oxygen MEFs (Fig. 2g). Thus, although MEFs that spontaneously immortalize in 20% oxygen frequently lose p53 function⁷,

most long-term MEF cultures in 3% oxygen retain p19^{ARF} and p53 regulation and function.

Similarly, the Rb pathway seemed to be intact in long-term 3% oxygen cultures. Growth in 3% oxygen did not increase levels of Cdk4 or Cdk2 (see Supplementary Information, Fig. S1b), which could, in principle, overcome the effects of high p16 levels (Fig. 2c; refs 2, 4). Moreover, serum withdrawal caused hypophosphorylation of Rb (Fig. 2h) and reduced DNA synthesis (see Supplementary Information, Fig. S1c) in all 3% oxygen cultures analysed. Hence, p16 and p19^{ARF} expression in 3% oxygen was not accompanied by obvious inactivation of Rb or p53. The ability to proliferate with high levels of p16 and p19^{ARF} may entail an adaptive response or mutation of an uncharacterized pathway that eventually allows proliferation in 20% oxygen (Fig. 2b).

Studies of cells from Balb/c mice, which are deficient in several DNA repair pathways, has provided evidence of why MEFs senesce in 20% oxygen^{17,18}. Balb/c MEFs had an extended replicative lifespan in 3% oxygen (Fig. 3a), but eventually senesced with a phenotype similar to other MEFs grown in 20% oxygen (see Supplementary Information, Fig. S2a, b). This extension of lifespan is unlikely to be caused by the Balb/c p16 polymorphism¹⁹, because *p16*-null MEFs senesce in 20% oxygen¹⁹, and, if the polymorphism conferred a proliferative advantage, Balb/c MEFs should proliferate longer than C57Bl/6 MEFs in 20% oxygen, which they did not (see Supplementary Information, Fig. S2c). Thus, low oxygen delayed, but did not abrogate, senescence of Balb/c MEFs, implicating DNA damage in the replicative arrest of MEFs.

To test this possibility, we studied MEFs from mice lacking Ku80 or the DNA-dependent protein kinase catalytic subunit (DNA-PKcs), which are essential for DNA double-strand break repair by non-homologous end joining²⁰. *Ku80*^{-/-} MEFs senesced rapidly in both 20% and 3% oxygen (Fig. 3b), whereas MEFs deficient in DNA-PKcs senesced rapidly in 20% oxygen, but grew continually in 3% oxygen (see Supplementary Information, Fig. S2d). Cytogenetic analysis (see Supplementary Information, Table S1) showed that absence of Ku80 or DNA-PKcs increased the frequency of chromosome fragments in 20% oxygen, as previously reported²¹. In 3% oxygen, however, *Ku80*^{-/-} cells showed many more chromosome fragments than DNA-PKcs-deficient MEFs. *Ku80*^{-/-} MEFs also accumulated more chromosome fusions than *DNA-PKcs*^{-/-}, but fusion frequencies were independent of oxygen tension. These data confirm that a deficiency of Ku80 results in more severe genomic aberrations than a deficiency of DNA-PKcs²¹, probably because Ku80 participates in other processes, such as telomere maintenance²¹. They also suggest that deficits other than low DNA-PK activity¹⁸ account for the finite lifespan of Balb/c MEFs in 3% oxygen. In contrast, *Xpa*^{-/-} MEFs²², which lack nucleotide excision repair (thought not to be important for repairing oxidative DNA lesions), grew similarly to wild-type MEFs in both 20% and 3% oxygen (Fig. 3c).

To understand the role of telomeres, we cultured MEFs from late-generation telomerase-null (*mTR*^{-/-}) mice. *mTR*^{-/-} MEFs senesced in 20% oxygen, as previously reported²³, but did not senesce in 3% oxygen (see Supplementary Information, Fig. S3b). Similarly to *Ku80*^{-/-} MEFs, *mTR*^{-/-} MEFs accumulated telomeric fusions in both 20% and 3% oxygen (see Supplementary Information, Table S1 and Fig. S3b). In contrast to *Ku80*^{-/-} MEFs, however, *mTR*^{-/-} MEFs accumulated significantly fewer chromosome breaks in 3% oxygen when compared with 20% oxygen (see Supplementary Information, Table S1), and in this regard were similar to wild-type cells (Fig. 4c; also see Supplementary Information, Table S1). These findings suggest that telomere dysfunction is not a major cause of MEF replicative senescence. Rather, oxidative DNA damage, and the breaks and/or chromosomal aberrations it can produce²⁴, is most probably responsible.

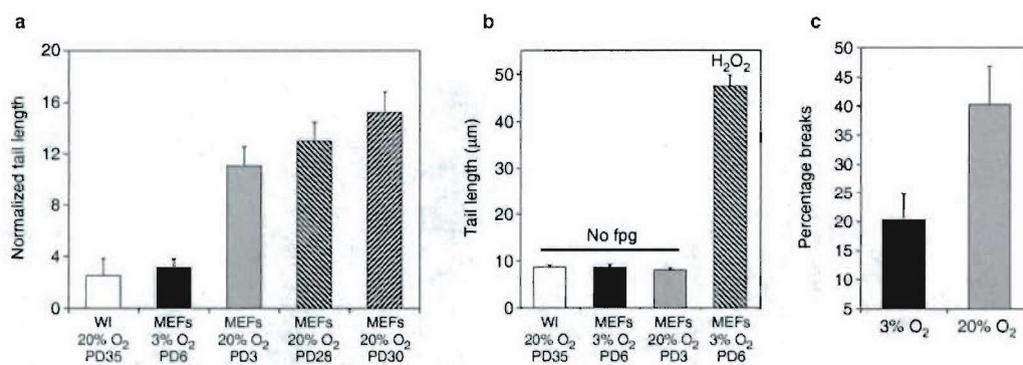


Figure 4 MEFs accumulate high levels of oxidative DNA damage in 20% oxygen. (a) The Fpg-comet assay was performed on early passage C57Bl/6 MEFs cultured in 3% or 20% oxygen, immortal MEFs cultures derived and grown in 20% oxygen and mid-lifespan (PD 35) WI-38 human fibroblasts cultured for more than 10 population doublings in 20% oxygen. The normalized average tail length is plotted. Each value represents the average of four independent experiments and at least 50 cells per determination. (b) Comet assay controls. WI38 and MEFs grown at the

indicated oxygen concentrations and at the indicated population doublings were analysed for comet tail lengths before treatment with fpg. As a positive control, the normalized tail length of MEFs treated with hydrogen peroxide (100 µM) is also shown. (c) C57Bl/6 MEFs were derived and grown in 3% or 20% oxygen for up to 25 passages. The average percentage of metaphases from 4–5 independent cultures containing obvious chromosomal breaks or fragments is plotted. Error bars show s.e.m.

To test this idea directly, we performed Fpg (formamidopyrimidine DNA glycosylase) comet assays, which measure oxidative DNA lesions²⁵. C57Bl/6 MEFs passaged in 20% oxygen for 10–14 days accumulated 3–4-fold more oxidative DNA damage than parallel cultures passaged in 3% oxygen (Fig. 4a). Little damage was detected in controls not treated with Fpg (Fig. 4b). Strikingly, MEFs cultured in 20% oxygen accumulated threefold more damage than human fibroblasts cultured in 20% oxygen. Thus, there was a major difference between mouse and human cells in relation to their ability to handle oxidative stress. Immortal MEFs that emerged in 20% oxygen also had substantial DNA damage (Fig. 4a). This suggests that immortalization in 20% oxygen, commonly caused by mutations in p53 or p19^{ARF} (ref. 7), does not prevent DNA damage, but rather renders cells insensitive to it. Finally, MEFs cultured in 20% oxygen showed twofold more chromosomal breaks than MEFs cultured in 3% oxygen (Fig. 4c; also see Supplementary Information, Table S1).

Our data indicate that the replicative senescence of MEFs is a consequence of severe oxidative stress, which causes extensive DNA damage. Although MEFs are telomerase-positive, this damage limits their

replicative lifespan, as the presence of telomerase cannot rescue cells from senescence caused by non-telomeric stimuli^{4,11,26}. Thus, MEFs proliferate indefinitely, as expected of telomerase-expressing cells²⁶ if severe oxidative damage is avoided. In addition, our finding that *mTR*^{-/-} MEFs do not senesce in 3% oxygen, in contrast to telomerase-negative human cells, is most probably caused by the ability of mouse chromosomes to stabilize telomeres in the absence of telomerase until alternative telomere maintenance pathways are induced^{23,27}. One stabilization mechanism is p-arm fusion to generate metacentric chromosomes, which allow late-generation *mTR*^{-/-} MEFs to undergo in excess of 250 population doublings in 20% oxygen^{23,27}. We detected metacentric chromosomes in *mTR*^{-/-} cultures, which may explain their failure to senesce in 3% oxygen.

In conclusion, our findings reconcile an apparent discrepancy in the behaviour of many mouse and human cells in culture. They also show that mouse and human cells differ markedly in their sensitivity to oxidative stress. This difference is manifest in their proliferative capacity and the steady state levels of DNA damage in 20% oxygen. We suggest

Table 1 MEFs cultured in 3% oxygen retain DNA damage checkpoints

PDs in 3% oxygen	Modification	Percentage of cells in S phase	
		Control	Control
5	None	15	5
24*	None	13	2
24*	None	15	3
5	pBABE	12	3
5	pBABE-GSE	14	19
36	PBABE	13	3
36	pBABE-GSE	15	16
38	pBABE	15	2
38	pBABE-GSE	13	12

C57Bl/6 MEFs were cultured in 3% oxygen for the indicated number of population doublings. Cells were either unmodified (none), infected with control retrovirus (pBABE) or infected with retrovirus expressing GSE-22 (pBABE-GSE), a dominant-interfering peptide that inactivates p53 function¹⁵. Cells were either mock-irradiated (control) or X-irradiated (4.5 Gy), then harvested 14 h later, stained with propidium iodide and analysed. *Two independent PD24 cultures were analysed.

that the superior ability of human cells to prevent or repair oxidative DNA damage contributes to the major differences in the incidence of cancer and the rate of ageing between mice and humans. □

METHODS

Mouse embryo fibroblast isolation and cell culture. *Ku80*^{-/-} (1295vEvBrd/C57Bl/6)²⁸, *DNA-PKcs*^{-/-} (C57Bl/6/129)²⁹, *mTR*^{-/-} (C57Bl/6/129)²³, *Xpa*^{-/-} (C57Bl/6)²², C57Bl/6, FVB and Balb/c mice were used to isolate MEFs as follows: torsos from 13.5-day embryos were washed and minced in 2 ml PBS using a syringe and an 18-gauge needle. After straining to remove large fragments, the suspension was placed in a 25-cm² flask containing DMEM, 10% foetal calf serum (FCS), 50 U ml⁻¹ penicillin and 50 µg ml⁻¹ streptomycin, buffered with bicarbonate and incubated in 10% CO₂ plus 3% or 20% oxygen, adjusted using an oxygen sensor and regulator and nitrogen source. After 2 days, cells that grew from tissue fragments were transferred to 75-cm² flasks and cultured to 90% confluency. From this enriched fibroblast population, 5 × 10⁵ cells were sub-cultured in 75-cm² flasks and considered as passage 1 and PD0. Each culture was derived from a single embryo and passaged at 5 × 10⁵ cells per 75 cm² every 3–4 days or at approximately 80% confluency. Cell number was determined and population doublings calculated at each passage, as previously described²⁶. Proliferative capacity was measured by labelling for 3 days with ³H-thymidine and counting labelled nuclei (percentage labelled nuclei)²⁶. At least two independent cultures and 500 cells were counted for each determination. Where indicated, sub-confluent cultures were incubated in DMEM containing 0.5% serum for 3–4 days and stimulated with DMEM containing 15% serum. Pre-senescent (percentage labelled nuclei, >65%) human fibroblasts were obtained and grown as described²⁶.

Hydrogen peroxide, bleomycin and X irradiation. Confluent pre-senescent cells were treated for 2 h with 0, 200, 400 or 550 µM hydrogen peroxide, or 20 µg ml⁻¹ bleomycin (Sigma, St Louis, MO) in DMEM containing 10% FCS. Cells were washed, incubated in fresh medium for 24 h and sub-cultured at 5 × 10⁵ cells per 75-cm² flask for 7 or 14 days.

Flow cytometry. Trypsinized cells were fixed in 75% ethanol and stored at 4 °C. After centrifugation, pellets were treated with 1 mg ml⁻¹ RNase A (Sigma) for 30 min at room temperature, washed with PBS and stained with propidium iodide (10 µg ml⁻¹) for 1 h. DNA content was analysed using a Beckman-Coulter (Miami, FL) EPICS XL fluorescence-activated cell sorter and Flowjo software. To assess DNA damage checkpoints, MEFs were processed before and 14 h after X irradiation (4.5 Gy).

Western blot analysis. Proteins (30 µg) were analysed by western blotting, as previously described¹⁰. Primary antibodies were M-156 for p16, pAb240 for p53, F-5 for p21 (Santa Cruz Biotechnology, Santa Cruz, CA), Ab-2 for c-Fos, Ab-1 for α -tubulin (Calbiochem, San Diego, CA), ab80 for p19^{ARF} (Abcam, Cambridge, UK), 610001 for Ras and G3-245 for Rb (Pharmingen, San Diego, CA). Secondary antibodies were detected by ECL or ECL plus (Amersham, Piscataway, NJ).

Retroviral infection. p-BABE-puro, pBABE-p19^{ARF}, pLXSN-p14^{ARF}, pBABE-GSE22 and pBABE-Ha-Ras^{G12V} vectors were used to produce retrovirus, as previously described²⁶. Proliferating cells (30–50% confluent) were infected for 8 h on two successive days, with a 16-h interval and medium change between infections. After 48 h, ³H-thymidine was added for 1 h and cells were processed for autoradiography, as described²⁶, or cells were selected in puromycin (2 µg ml⁻¹) for 4–5 days and expanded in antibiotic-free medium. Lysates were obtained 14 days after infection.

Comet assay. An Fpg-FLARE (fragment length analysis using repair enzymes) comet assay kit was used in accordance with the manufacturer's instructions (Trevigen, Gaithersburg, MO). This kit specifically detects oxidative DNA lesions such as 8-oxo-2'-deoxyguanosine and formamidopyrimidines. Images of 50 randomly chosen nuclei per sample were captured using a CCD camera coupled to an epifluorescence microscope. Comet tail lengths were measured using the comet macro from the NIH public domain image analysis program³⁰. For each experimental point, four independent cultures were analysed. Average tail lengths were calculated for buffer controls and Fpg-treated samples.

Oxidative DNA damage was estimated by subtracting the mean tail length of the control from that of the Fpg-treated sample (normalized mean tail length).

Cytogenetic analysis. MEFs were treated with colcemid (0.1 µg ml⁻¹) for 4 h, trypsinized and centrifuged at 120g for 8 min. After hypotonic swelling in sodium citrate (0.03 M) for 25 min at 37 °C, the cells were gradually fixed in methanol:acetic acid (3:1). Cell suspensions were dropped onto clean wet slides and dried overnight. Fluorescence *in situ* hybridization (FISH) with a Cy3-labelled (CCCTAA)₃ probe (Applied Biosystems, Foster City, CA) was performed as described²¹. Cy3 and DAPI images from 50 metaphases were acquired with a Zeiss Axioplan2 epifluorescence microscope (Zeiss, Thornwood, NJ) and Axiocam colour digital camera, and analysed in a blinded fashion. The telomeric Cy3 image was superimposed on the DAPI image to accurately score chromosomal aberrations, as described²¹, using the ImageJ software package (<http://rsb.info.nih.gov/ij>).

Note: Supplementary Information is available on the Nature Cell Biology website.

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COMPETING FINANCIAL INTERESTS

The authors declare that they have no competing financial interests.

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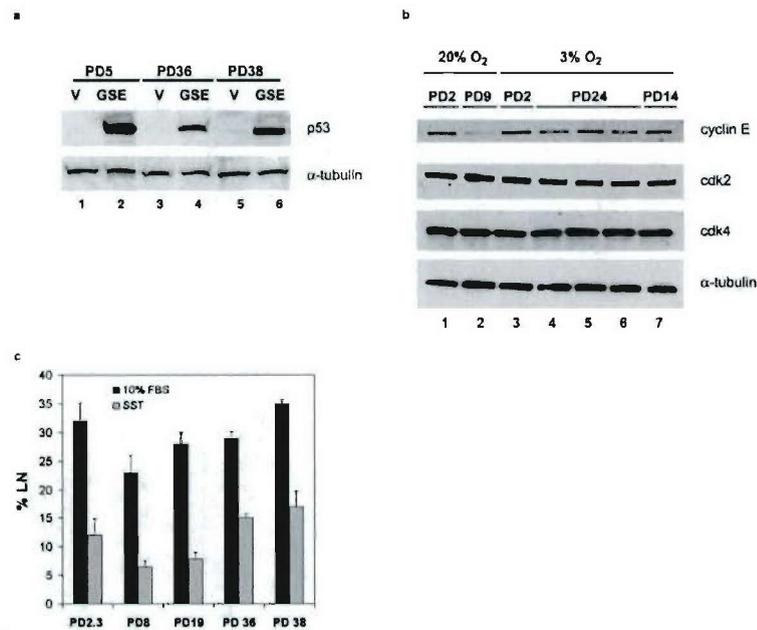


Figure S1. Efficacy of GSE, levels of G₁ cyclin-dependent kinases, and responsiveness to serum deprivation of MEFs cultured in 3% O₂. **a**, An early passage (PD5) and two late passage (PD36 and PD38) C57Bl/6 MEF cultures grown in 3% O₂ were infected with insertless (lanes 1, 3, 5) or GSE-expressing (lanes 2, 4, 6) pBABE-puro retroviruses. Proteins were prepared and assayed for p53 and α -tubulin (control) by western blotting. **b**, Proteins were prepared from C57Bl/6 MEFs proliferating in 20% (lanes 1, 2) or 3% (lanes 3-7) O₂, and analyzed

by western blotting for cyclin E, Cdk2, Cdk4 and α -tubulin. Early passage (PD 2, lane 1) and senescent (PD 9, lane 2) cultures in 20% O₂, and early (PD 2, lane 3), mid- (PD 14, lane 7), and three independent later (PD 24, lanes 3-6) passage 3% O₂ cultures were analyzed. **c**, Five MEF cultures were grown for the indicated PDs in 3% O₂. The cells were shifted to 10% (10% FBS; black bars) or 0.5% (SST; gray bars) serum for 3 d before ³H-thymidine was added for 1 h. The fraction of cells synthesizing DNA was determined from the %LN.

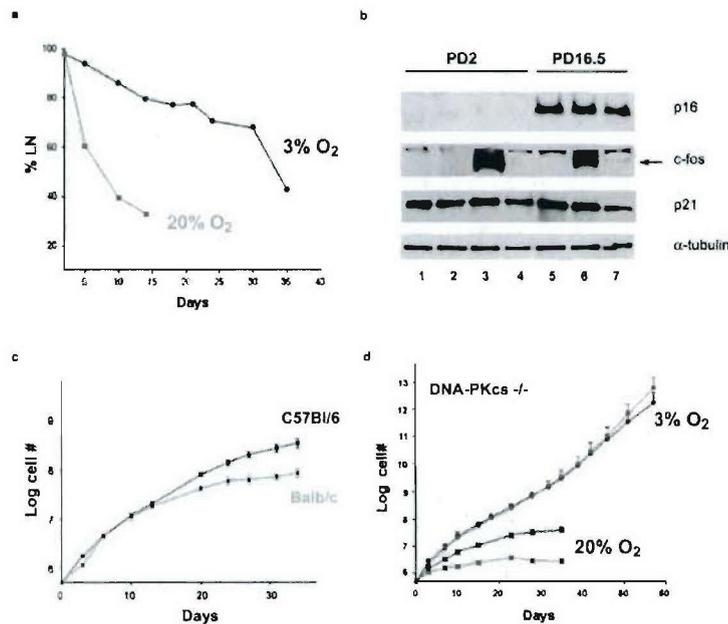


Figure S2. Senescence of Balb/c and DNA-PKcs^{-/-} MEFs in low oxygen. **a**, The labeling index (% LN) was determined at every passage for representative Balb/c cultures grown in 20% (gray) or 3% (black) O₂. **b**, Early passage (PD 2, lanes 1-4) and senescent (PD 16.5, lanes 5-7) Balb/c MEF cultures grown in 3% O₂ were analyzed for C-FOS, p16, p21 and α -tubulin by western blotting. Proteins were prepared from exponentially growing (lane 1) and serum-deprived (lanes 2 and 5)

cultures, or cultures that were serum deprived and then serum-stimulated for 1.5 h. (lanes 3, 6) or 6 h (lanes 4, 7). **c**, The lifespan of three C57Bl/6 (black) and three Balb/c (gray) MEF cultures, derived and grown in 20% O₂, was determined. **d**, The replicative life span of DNA-PKcs^{-/-} MEFs in 3% or 20% O₂ is shown. Four DNA-PKcs (gray) and three wild-type littermate (black) cultures were derived and grown in 3% or 20% O₂.

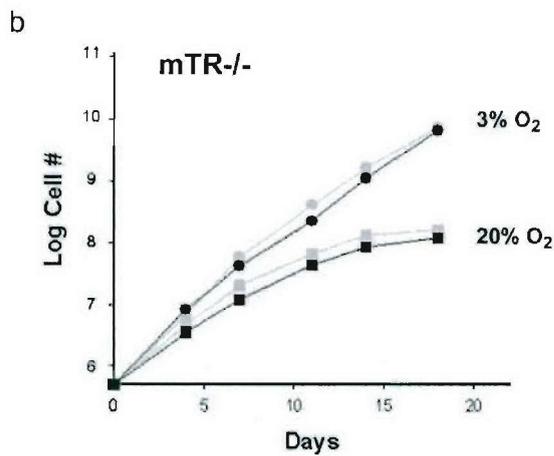
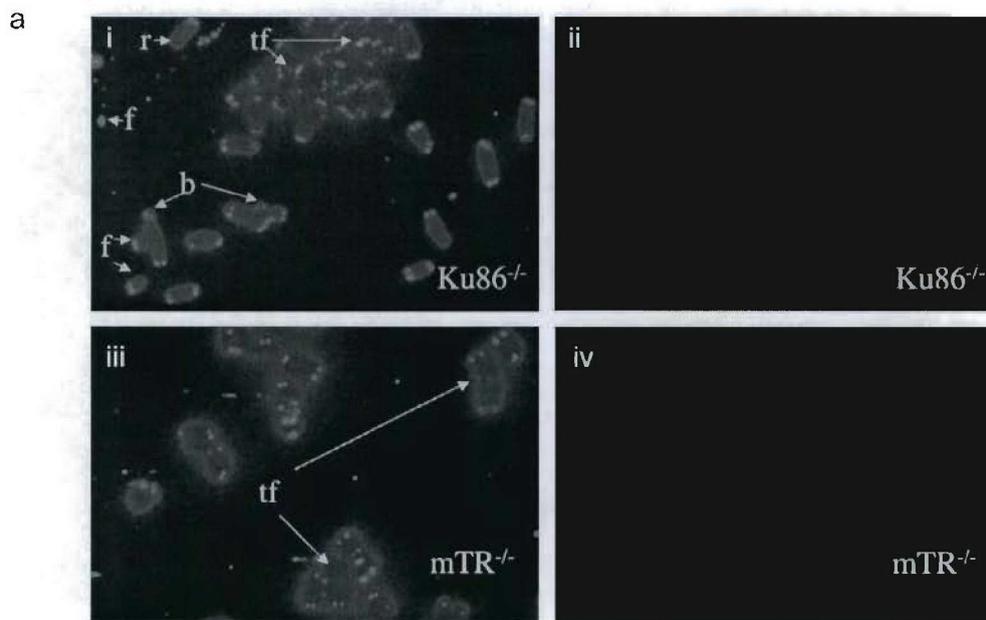


Figure S3. **Telomere-FISH and growth of telomerase-deficient MEFs in 3% and 20% O₂.** **a**, Representative metaphase spreads of early passage *ku80*^{-/-} and fourth generation *mTR*^{-/-} MEFs before (ii and iv) and after (i and iii) fluorescence *in situ* hybridization (FISH) with a telomere probe. Arrows denote breaks (b).

fragments (f), rings (r) and telomeric fusions (tf). **b**, Replicative life spans of a representative fourth generation *mTR*^{-/-} (gray) and wild-type littermate (black) MEF culture in 3% O₂ (circles) and 20% (squares) O₂ are shown. Duplicate cultures were grown in parallel. Similar results were obtained with an independent *mTR*^{-/-} MEF culture.

Stromal-epithelial interactions in aging and cancer: senescent fibroblasts alter epithelial cell differentiation

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Summary

Cellular senescence suppresses cancer by arresting cells at risk of malignant tumorigenesis. However, senescent cells also secrete molecules that can stimulate premalignant cells to proliferate and form tumors, suggesting the senescence response is antagonistically pleiotropic. We show that premalignant mammary epithelial cells exposed to senescent human fibroblasts in mice irreversibly lose differentiated properties, become invasive and undergo full malignant transformation. Moreover, using cultured mouse or human fibroblasts and non-malignant breast epithelial cells, we show that senescent fibroblasts disrupt epithelial alveolar morphogenesis, functional differentiation and branching morphogenesis. Furthermore, we identify

MMP-3 as the major factor responsible for the effects of senescent fibroblasts on branching morphogenesis. Our findings support the idea that senescent cells contribute to age-related pathology, including cancer, and describe a new property of senescent fibroblasts – the ability to alter epithelial differentiation – that might also explain the loss of tissue function and organization that is a hallmark of aging.

Key words: Epithelial to mesenchyme transition (EMT), Beta-casein, Mammary epithelial cells, Matrix metalloproteinase-3 (MMP-3), Morphogenesis, Tissue structure and function

Introduction

The incidence of cancer rises exponentially with age among mammalian species. In humans and other non-inbred species, most age-related cancers arise from epithelial cells (DePinho, 2000; Repetto and Balducci, 2002). Somatic mutations, which accumulate throughout life, are a major contributor to this age-dependent increase in epithelial tumors (DePinho, 2000; Dolle et al., 2002). However, several lines of evidence suggest that mutations alone are insufficient for the development of cancer (Bissell and Radisky, 2001; DePinho, 2000; Krtolica and Campisi, 2002). Rather, malignant tumorigenesis also requires a permissive tissue microenvironment in which cells that bear potentially oncogenic mutations can progress towards full malignancy. Fully malignant cancer cells typically acquire an array of malignant properties, among which are loss of proper growth control and aberrant differentiation.

Two cellular tumor suppressor mechanisms – apoptosis and senescence – limit the proliferation (used here interchangeably with growth) of cells at risk of malignant transformation (Campisi, 2003). Apoptosis, or programmed cell death, eliminates potential cancer cells, whereas cellular senescence prevents their proliferation by imposing an irreversible block to cell cycle progression. The senescence response was first formally described as the process that limits the proliferation of normal human cells in culture (Hayflick, 1965). This limit is now known to be due, in large measure, to the progressive

telomere shortening (and subsequent dysfunction) that occurs when cells undergo DNA replication in the absence of telomerase (Aisner et al., 2002; Kim et al., 2002). In addition to telomere dysfunction, a variety of other potentially oncogenic events or stimuli elicit a senescence response. These include direct DNA damage, the expression of certain oncogenes and epigenetic perturbations in chromatin organization (Chen et al., 1995; DiLeonardo et al., 1994; Krtolica and Campisi, 2002; Ogryzko et al., 1996; Robles and Adami, 1998; Serrano et al., 1997).

Senescent cells acquire multiple phenotypic changes, in addition to an irreversible growth arrest, some of which can compromise tissue structure and function (reviewed by Krtolica and Campisi, 2002). These findings have led to the hypothesis that the senescence response is an example of evolutionary antagonistic pleiotropy. Thus, this response may benefit organisms early in life by preventing cancer, but be detrimental later in life as senescent cells accumulate (Campisi, 2003; DePinho, 2000; Rinehart and Torti, 1997). Senescent cells may be detrimental not only because they compromise tissue renewal capacity, but also because they secrete factors that alter tissue homeostasis. For example, senescent stromal fibroblasts secrete soluble and insoluble factors that can, at least in principle, disrupt the architecture and function of the surrounding tissue and stimulate (or inhibit) the proliferation of neighboring cells. These factors include inflammatory

cytokines (e.g. IL1), epithelial growth factors (e.g. heregulin) and matrix metalloproteinases (e.g. MMP-3) (Krtolica and Campisi, 2002). Thus, senescent cells may create a tissue environment that synergizes with mutation accumulation to facilitate the progression of epithelial malignancies (Campisi, 2003; DePinho, 2000; Rinehart and Torti, 1997). Consistent with this idea, human and rodent cells with senescent characteristics accumulate *in vivo* with age and at sites of age-related pathology, including hyperplastic and premalignant lesions (Choi et al., 2000; Dimri et al., 1995; Krtolica and Campisi, 2002; Melk et al., 2003; Paradis et al., 2001; Vasile et al., 2001). Moreover, senescent human fibroblasts can promote the proliferation and tumorigenic conversion of premalignant (non-tumorigenic, but bearing potentially oncogenic mutations), but not normal, epithelial cells in culture and *in vivo* (Krtolica et al., 2001).

We recently showed that immortal but non-tumorigenic mammary epithelial cells (SCp2), which express cytokeratins and functionally differentiate in culture (Desprez et al., 1998), produce undifferentiated tumors when injected into mice together with senescent, but not presenescent, fibroblasts (Krtolica et al., 2001). Notably, the resulting tumors were devoid of cytokeratin expression, one sign of an epithelial-to-mesenchymal-transition (EMT). The EMT is a phenotypic switch that enables preneoplastic epithelial cells to acquire more malignant properties, specifically the ability to migrate and invade the basement membrane (Birchmeier and Birchmeier, 1995). Because loss of differentiation is a hallmark of cancer progression (Bissell and Radisky, 2001; Petersen et al., 1998), this finding suggested that senescent fibroblasts might promote age-related cancer in part by altering epithelial differentiation.

Epithelial cells from the mammary gland have provided numerous insights into the control of cell proliferation and differentiation and their relationship to carcinogenesis. The mammary gland consists of branched epithelial ducts that culminate in secretory alveoli, the functional milk-producing units of the gland. The mammary parenchyma is embedded in a stroma composed of an extracellular matrix (ECM) and a variety of cell types, including adipocytes and fibroblasts (mesenchyme). All stages of mammary gland development depend on epithelial-stromal interactions (Fata et al., 2004; Woodward et al., 1998). During both branching and alveolar differentiation, the mammary mesenchyme synthesizes factors that, in concert with systemic hormones, direct epithelial function (Woodward et al., 1998).

We present evidence that senescent fibroblasts influence both the morphological and functional differentiation of mammary epithelial cells. Our findings support the idea that senescent stromal cells might promote malignant transformation in part by altering epithelial differentiation. They also suggest that senescent cells might contribute to the decline in tissue function that is a hallmark of mammalian aging.

Materials and Methods

Cells

WI-38 fetal lung (ATTC) and 48 adult breast human fibroblasts (hBF) [M. Stampfer, P. Yaswen, Lawrence Berkeley National Laboratory (LBNL), Berkeley, CA], which senesce after ~50 and 25 population

doublings (PDs), respectively, were cultured as described previously (Dimri et al., 1995). Presenescent and senescent cultures contained, respectively, >60% and <10% proliferating cells and <10% and >60% senescence-associated β -galactosidase (SA- β gal)-positive cells (Dimri et al., 1995). SCp2-P, SCp2-T, EpH4 and MCF-10A (M. Bissell, LBNL, Berkeley, CA) cells were cultured as described previously (Desprez et al., 1998; Montesano et al., 1998). Mouse mammary fibroblasts (mBF) and mammary epithelial organoids were isolated from 3- to 4-month-old virgin C57Bl/6 mice and cultured as described previously (Simian et al., 2001), with the exception that they were cultured in a 3% oxygen atmosphere (Parrinello et al., 2003).

Inhibitors and reagents

HGF neutralizing antibody (2.5 μ g/ml) and insulin-like growth factor neutralizing antibody (10 μ g/ml) were from Sigma. The EGF neutralizing antibody (20 μ g/ml) was from Upstate Biotechnologies. MMP-3 inhibitor I (25 μ M), MMP-2 inhibitor I (8.5 μ M) and human MMP-3 catalytic domain (0.5–2 μ M) were from Calbiochem.

Conditioned media

Fibroblasts were plated on 60 mm dishes and cultured until they reached confluence. Confluent cultures were rinsed twice with serum-free medium and incubated in basal branching medium [phenol red-free DMEM/F12 (Sigma), 0.1 mM non-essential amino acids (Gibco), 2 mM L-glutamine (Gibco), 100 ng/ml insulin (Sigma), 1 mg/ml fatty acid-free BSA (fraction V) (Sigma), 0.5 mg/ml fetuin (Sigma)] for 48 hours. We collected the conditioned media and clarified them by centrifugation, counted the number of cells on the dish, and normalized the volume of conditioned medium used in each experiment for cell number. Inhibitors and antibodies described above were added to conditioned media for 2 hours at 37°C prior to addition to cell cultures.

Induction of senescence

Confluent presenescent fibroblasts were irradiated with 10 Gy X-rays, incubated in fresh medium for 24 hours, then trypsinized and replated at subconfluent densities. After replating, the cells acquired a senescent-like phenotype within 7 days. The senescence response was confirmed by determining the percentage of cells capable of DNA synthesis over a 3-day interval (3 H]thymidine incorporation; %LN) and expression of SA- β gal, as described previously (Dimri et al., 1995).

Tumorigenesis assays

Tumorigenesis assays were performed as described previously (Krtolica et al., 2001). Tumors (~100 mm³) were excised aseptically and digested with 0.2% trypsin (Invitrogen), 0.2% collagenase, 5% fetal bovine serum (FBS), 5 μ g/ml insulin and 50 μ g/ml gentamicin (Sigma) in DME/F12 (Invitrogen) for 30 minutes. The dispersed tumor cells were separated through Percoll gradients (Redigrad, Amersham Biosciences) according to manufacturer's instructions. The epithelial layer was transferred to 10 cm² dishes and cultured as described for parental SCp2 cells (Desprez et al., 1998; Montesano et al., 1998). Parental and tumor cells (3×10^4) were embedded in 60 μ l undiluted Matrigel (BD Biosciences) in 4-well chamber slides (Nunc) and allowed to differentiate over 5–7 days, as described previously (Desprez et al., 1998).

Three-dimensional (3D) co-cultures

Presenescent or senescent hBF (1.2 or 1.8×10^4 /well, respectively) were suspended in 60 μ l cold Matrigel and plated in 24-well plates (Corning) or 4-well chamber slides. After the Matrigel gelled (37°C,

45 minutes), MCF-10A or Eph4 cells (3×10^4 /well) were plated on top of the fibroblast-Matrigel layer. MCF-10A cells were plated in MEGM medium supplemented with bovine pituitary extract, insulin, hydrocortisone and EGF (Clonetics). Eph4 cells were plated in differentiation medium [DME/F12, 5 μ g/ml insulin, 1.4 μ M hydrocortisone, 3 μ g/ml prolactin (Sigma)] containing 10% FBS; after 24 hours, the cells were given serum-free differentiation medium. The cultures were maintained at 37°C in a 5% CO₂ and 3% O₂ atmosphere for up to 6 days.

Branching assays

Presenescent (PD 2) or senescent (PD 2, X-irradiated) mBF (10^5 or 1.2×10^5 /well) were cultured in the lower well of Corning clear transwell inserts in 24-well plates. Mammary epithelial organoids (80/well) suspended in type I collagen (BD Biosciences; 2 mg/ml, 110 μ l/well) were plated in the upper well of the transwells. The collagen was allowed to gel for 30 minutes at 37°C and basal branching medium (described above under Conditioned media) was added. Alternatively, the epithelial cells in collagen were plated in 96-well plates, and fibroblast conditioned medium was added. The cultures were maintained at 37°C in a 5% CO₂ and 3% O₂ atmosphere for 4 days. Fresh culture or conditioned medium was added every 48 or 24 hours, respectively.

Quantification of branching morphogenesis

Phase-contrast images were captured at 100 \times magnification using a Spot camera. For each experimental condition, a minimum of 25 organoids were randomly chosen from triplicate culture wells and analyzed for the size, number, type and length of branches using Spot software. Alternatively, 15 organoids randomly chosen from triplicate wells were scored visually for the presence and extent of branching and classified accordingly. To quantify proliferation, cultures were fixed with ice-cold methanol for 20 minutes and stained with DAPI (4',6'-diamidino-2-phenylindole; Sigma), as described previously (Krtolica et al., 2001). Fluorescent images of the DAPI-stained nuclei from at least 25 organoids from triplicate wells were quantified using NIH image common domain software.

Immunocytochemistry and western blotting

Matrigel-embedded cells and 3D co-cultures in chamber slides were fixed with 4% formaldehyde for 20 minutes, permeabilized, blocked with 10% FBS for 30 minutes, then incubated with primary antibodies overnight followed by incubation with FITC- or Texas Red-conjugated secondary antibodies (Alexa) for 1 hour. Nuclei were counterstained with DAPI. The slides were mounted in VectaShield (Vector Laboratories) and viewed by epifluorescence. Primary antibodies were: β -casein (1:500 or 1:3000; a gift from Mina Bissell), cytokeratin 18 (1:500; Sigma), pan-cytokeratin (1:100; DAKO), vimentin (1:200; Sigma), $\alpha 6$ -integrin (1:200; Chemicon), E-cadherin (1:100) and GM130:FITC (1:250) (BD Transduction Labs), and Ki67 (1:500; Novo Castra).

Boyden chamber invasion assays

Invasion assays were performed in modified Boyden chambers with 8 mm pore-size filter inserts (Corning), as described previously (Desprez et al., 1998). After 20 hours, cells on the lower side of the filter were fixed with 2.5% glutaraldehyde, stained with crystal violet, and counted.

Zymography

Confluent presenescent or senescent fibroblasts (1.2×10^6 in 60-mm dishes) were shifted to serum-free medium for 2 days, after which the

conditioned medium was collected and concentrated 10- to 15-fold using 30 kDa cutoff filters (Millipore, Bedford, MA). The concentrated conditioned medium was analyzed on casein substrate gels as described by Desprez et al. (Desprez et al., 1998), normalizing to cell number at the time of medium collection.

Results

Phenotype of epithelial tumor cells induced by senescent fibroblasts

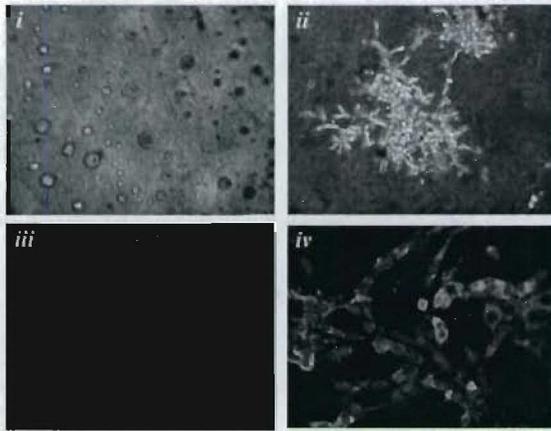
The immortal but non-tumorigenic mouse mammary epithelial cell line SCp2 formed tumors only in the presence of senescent fibroblasts (human WI-38, from fetal lung) (Krtolica et al., 2001). Moreover, the tumor cells, in contrast to the parental SCp2 cells, were devoid of cytokeratin expression. Experiments using green fluorescent protein (GFP)-expressing epithelial cells showed senescent fibroblast-induced tumors arose from the injected epithelial cells, not from the injected fibroblasts or host cells (Krtolica et al., 2001).

To better understand this tumorigenic conversion, we injected into mice the parental SCp2 cells (SCp2-P) together with replicatively senescent human fibroblasts (WI-38). We allowed tumors to develop, excised the tumors and dissociated the tumor cells. We then isolated and cultured the tumor epithelial cells, which we term SCp2-T cells. We then used the Scp2-T cells to determine whether their malignant properties remained dependent on the presence of senescent fibroblasts or were autonomous of a senescent stroma.

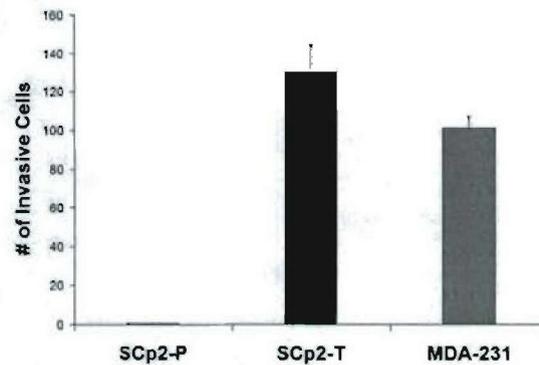
Non-malignant breast epithelial cells can be distinguished from their malignant counterparts by the size and organization of structures they form in three-dimensional (3D) cultures using a basement membrane-like ECM such as Matrigel (Petersen et al., 1992). Thus, we first compared the behavior of SCp2-P and SCp2-T cells in basement membrane-rich 3D cultures. As reported (Desprez et al., 1998), when cultured in 3D in Matrigel, SCp2-P cells formed small, uniform, well-organized structures that resembled differentiated alveoli (Fig. 1A*i*). In contrast, SCp2-T cells formed large, irregular, highly disorganized structures with numerous elongated cells and protrusions (Fig. 1A*ii*). Thus, upon tumorigenic conversion by senescent fibroblasts *in vivo*, SCp2 cells lost the ability to respond to signals for morphological differentiation and behaved similarly to frankly malignant cells, and these phenotypic changes were independent of the presence of senescent fibroblasts.

The elongated spindle shape of SCp2-T cells in Matrigel resembled that of aggressive breast cancer cells (Petersen et al., 1992), which frequently have undergone an EMT. To explore the possibility that Scp2 cells underwent an EMT during tumorigenic conversion by senescent fibroblasts *in vivo*, we immunostained the structures formed by SCp2-T cells in Matrigel for cytokeratin and vimentin filaments. In contrast to the cytokeratin-positive and vimentin-negative parental (SCp2-P) cells (Fig. 1A*iii*), SCp2-T cells stained weakly for cytokeratin and strongly for vimentin (Fig. 1A*iv*). In addition, SCp2-T cells were highly invasive, in sharp contrast to non-invasive Scp2-P cells. To demonstrate this, we seeded SCp2-P and SCp2-T cells in the upper wells of Boyden chambers (Albini et al., 1987), placed an attractant (NIH3T3 fibroblast-conditioned medium) in the lower wells, and coated

A



B



C

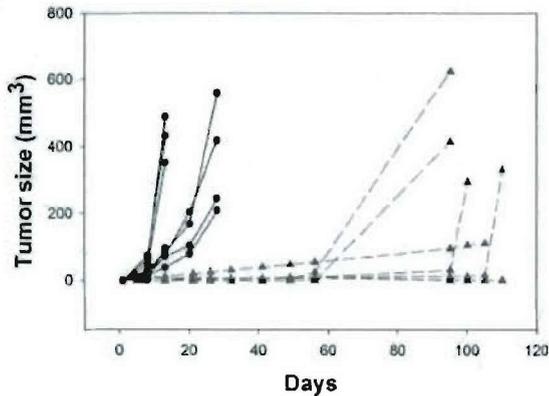


Fig. 1. Epithelial tumors stimulated by senescent human fibroblasts progress to full malignancy. (A) (*i* and *ii*) Morphology (40 \times magnification), and (*iii* and *iv*) cytokeratin (red) and vimentin (green) immunostaining (400 \times magnification), of SCp2-P (*i* and *iii*) and SCp2-T (*ii* and *iv*) cells in 3D Matrigel culture. (B) Invasion and migration of SCp2-P, SCp2-T and MDA-MB-231 (positive control) cells through basement membrane components (Matrigel) in Boyden chamber assays. Error bars indicate the s.e.m. from three fields. (C) Tumorigenicity of SCp2-T cells injected alone (solid black lines) into immunocompromised mice. Tumor cells were injected and tumor size was measured at the indicated intervals after injection. Tumors formed by SCp2-P cells co-injected with senescent fibroblasts (gray lines) is shown for comparison (Krtolica et al., 2001).

the porous filter separating the wells with Matrigel. After 20 hours, we fixed, stained, and counted the number of cells that invaded and migrated through the Matrigel to the underside of the filter. As expected (Desprez et al., 1998), SCp2-P cells did not migrate through the filter (Fig. 1B). By contrast, SCp2-T cells were decidedly migratory and invasive, behaving similarly in this regard to the highly malignant human breast cancer cell line MDA-MB-231, which served as a positive control (Fig. 1B).

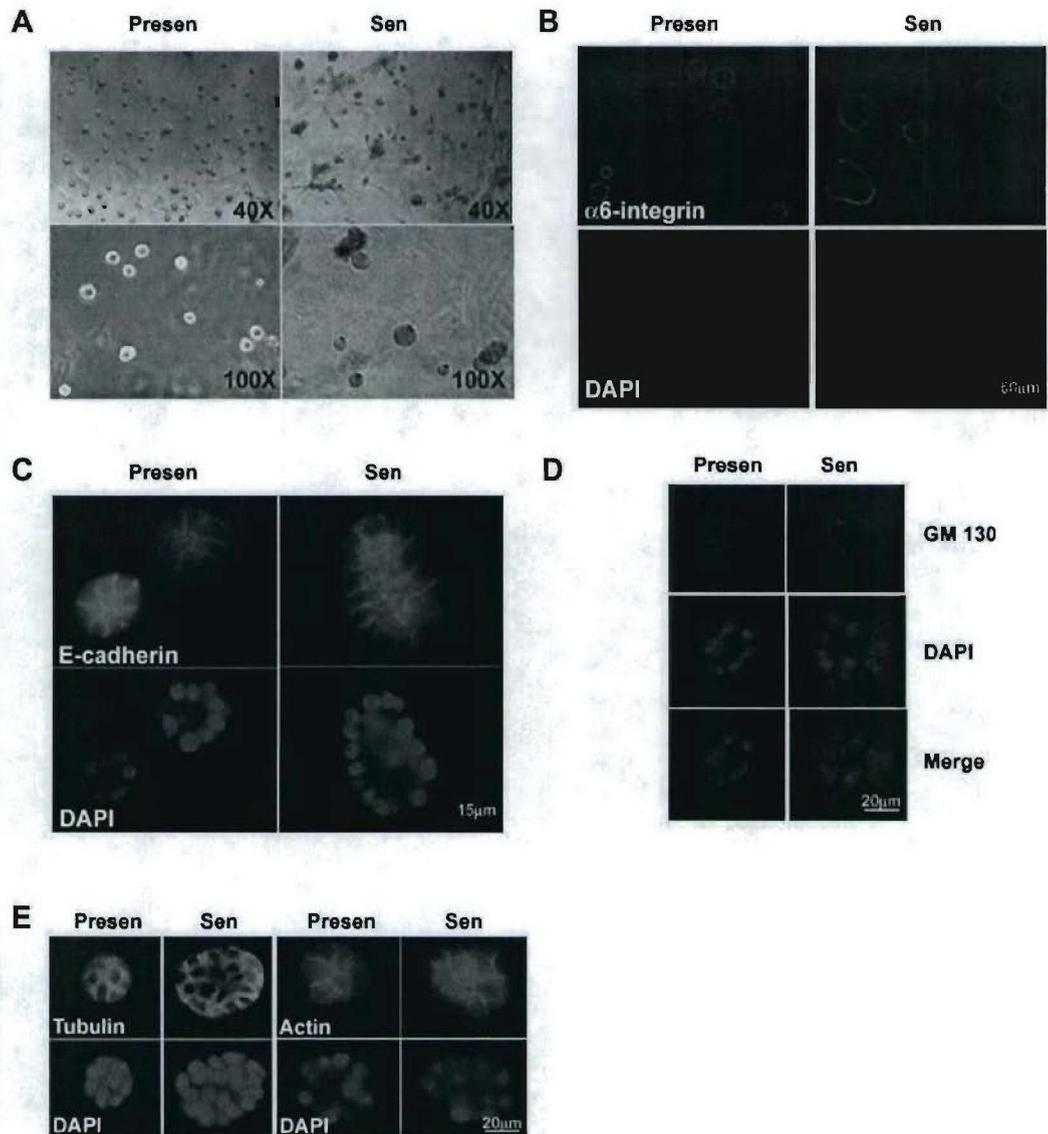
The aggressive properties conferred on SCp2 cells by senescent fibroblasts did not depend on their continuous presence. We injected SCp2-T cells alone into mice. In contrast to the long latency required for tumors produced by SCp2-P cells plus senescent fibroblasts (Fig. 1C, gray lines), SCp2-T cells alone produced large tumors with a very short latency period (Fig. 1C, black lines). Although we cannot rule out the possibility that senescent fibroblasts favored the outgrowth of rare vimentin-positive cells, which occur spontaneously at low frequency in SCp2 populations (Desprez et al., 1998), taken together, these results suggest that senescent fibroblasts can induce an essentially irreversible progression towards aberrantly differentiated, highly invasive malignant phenotypes, possibly by inducing an EMT.

Senescent fibroblasts disrupt morphological and functional differentiation of non-malignant epithelial cells

To study the effect of senescent fibroblasts on mammary epithelial differentiation more directly, we established homotypic 3D co-cultures using human breast fibroblasts (hBF), together with two immortal but non-tumorigenic (human or mouse) breast epithelial cell lines. For the remainder of our studies, we used breast fibroblasts in order to recapitulate as closely as possible the physiological epithelial-stromal interactions in the mammary gland. In addition, we used presenescent fibroblasts that were either untreated or induced to senesce by X-irradiation (DiLeonardo et al., 1994; Robles and Adami, 1998). Recent studies in our laboratory showed that there are no major differences in the secretory profiles of fibroblasts induced to senesce by replicative exhaustion or X-irradiation (our unpublished work). Moreover, DNA damage is probably an important contributor to the accumulation of senescent cells *in vivo* (Hasty et al., 2003). Senescence was confirmed in cells by the characteristic senescent morphology, long term reduction in the labeling index, and expression of SA- β gal (Dimri et al., 1995).

We first used MCF-10A cells, a human breast epithelial cell line that forms branched tubular networks when cultured on Matrigel (Shekhar et al., 2001) and polarized alveoli when

Fig. 2. Senescent human breast fibroblasts attenuate mammary epithelial alveolar differentiation. (A) MCF-10A cells co-cultured with presenescent (Presen, left) or senescent (Sen, right) hBFs in 3D on Matrigel. Low (40 \times , upper panels) and high (100 \times , lower panels) magnifications of the cultures are shown. (B) $\alpha 6$ integrin immunostaining (green) of MCF-10A cells co-cultured on Matrigel with presenescent (Presen, left) or senescent (Sen, right) hBFs. Nuclei were counterstained with DAPI (blue); images shown at 200 \times magnification. (C) E-cadherin immunostaining (green) of MCF-10A cells co-cultured on Matrigel with presenescent (Presen, left) or senescent (Sen, right) hBFs. Nuclei were counterstained with DAPI (blue); images shown at 1000 \times magnification. (D) GM-130 immunostaining (green) of a representative MCF-10A alveolus co-cultured on Matrigel with presenescent (Presen, left) or senescent (Sen, right) hBFs. Nuclei were counterstained with DAPI (blue). Merged images show the apical polarity of GM130; images shown at 400 \times magnification. (E) Tubulin (green) and actin (red) immunostaining of representative MCF-10A alveoli co-cultured on Matrigel with (Presen, left) or senescent (Sen, right) hBFs. Nuclei were counterstained with DAPI (blue), and images shown at 400 \times magnification.



cultured in Matrigel (Soule et al., 1990). As expected, when cultured on Matrigel alone, MCF-10A cells formed branched tubular networks or single cells that did not proliferate (not shown). However, when cultured on Matrigel containing hBFs, MCF-10A formed alveolar structures (Fig. 2A). Regardless of whether the fibroblasts were presenescent or senescent (%LN=61% for untreated hBF and 3% for X-irradiated hBF), the MCF-10A alveoli were composed of cytokeratin-positive cells (not shown). Moreover, the alveoli were polarized, as judged by immunostaining for basally localized $\alpha 6$ -integrin, laterally localized E-cadherin and apically localized GM130, a Golgi marker (Fig. 2B-D). Cytoskeletal organization was also similar in cultures containing presenescent or senescent fibroblasts, as evidenced by tubulin and actin immunostaining (Fig. 2E). However, alveoli that formed in the presence of senescent fibroblasts were less uniform and often larger than those formed in the presence of presenescent fibroblasts (Fig.

2A-E, Fig. 3A,C). This larger alveolar size was due at least in part to increased cell proliferation, as determined by the number of cells positive for Ki67 staining (Fig. 3A,B). After 4 days in 3D culture, only 20% of the alveoli in cultures with presenescent fibroblasts contained >5 Ki-67-positive nuclei; by contrast, >50% of the alveoli in cultures containing senescent fibroblasts had >5 Ki67-positive nuclei (Fig. 3B). Alveoli formed in the presence of senescent hBFs were on average two-fold larger than alveoli formed in the presence of presenescent hBFs (Fig. 3C).

Lobuloalveolar development of the mammary gland culminates in milk production (lactation), the functionally differentiated state of mammary epithelial cells. This differentiation requires both hormonal and ECM-mediated signals (Rosen et al., 1999), as well as signals from surrounding stromal fibroblasts (Darcy et al., 2000).

To assess the effect of senescent fibroblasts on functional

differentiation, we used the immortal but non-tumorigenic mouse mammary epithelial cell line Eph4. When cultured on Matrigel with lactogenic hormones, Eph4 cells form alveoli and additionally, unlike MCF-10A cells, express milk proteins (Montesano et al., 1998). We plated Eph4 cells on Matrigel containing presenescent or senescent hBFs, then maintained the cultures in serum-free medium containing lactogenic hormones. After 6 days, Eph4 cells formed alveolar structures, regardless of whether the fibroblasts were presenescent or senescent. However, many of the Eph4 alveoli that formed in the presence of senescent fibroblasts, like similarly formed MCF-10A alveoli, were on average larger, less uniform and less organized than those formed in the presence of presenescent fibroblasts (Fig. 3E). Moreover, the alveoli formed in the presence of senescent fibroblasts expressed 1.6- to 2-fold less β -casein, a major milk protein, as determined by

western blotting (Fig. 3D) and immunostaining (Fig. 3E). Western blots were normalized to cytokeratin 18, which luminal mammary epithelial cells express independently of functional differentiation (Smalley et al., 1999). We also confirmed by immunostaining that cytokeratin 18 was expressed at similar levels in both presenescent and senescent co-cultures (not shown). A similar senescent fibroblast-induced reduction in β -casein expression was observed when cytokeratin 8 was used to normalize the western blots (not shown).

Taken together, these results suggest that senescent human fibroblasts can impair the morphological (alveolar) and functional differentiation of human and mouse mammary epithelial cells. Moreover, this impairment is due in part to the ability of senescent fibroblasts to stimulate the proliferation of epithelial cells in 3D co-cultures (Krtolica et al., 2001). In

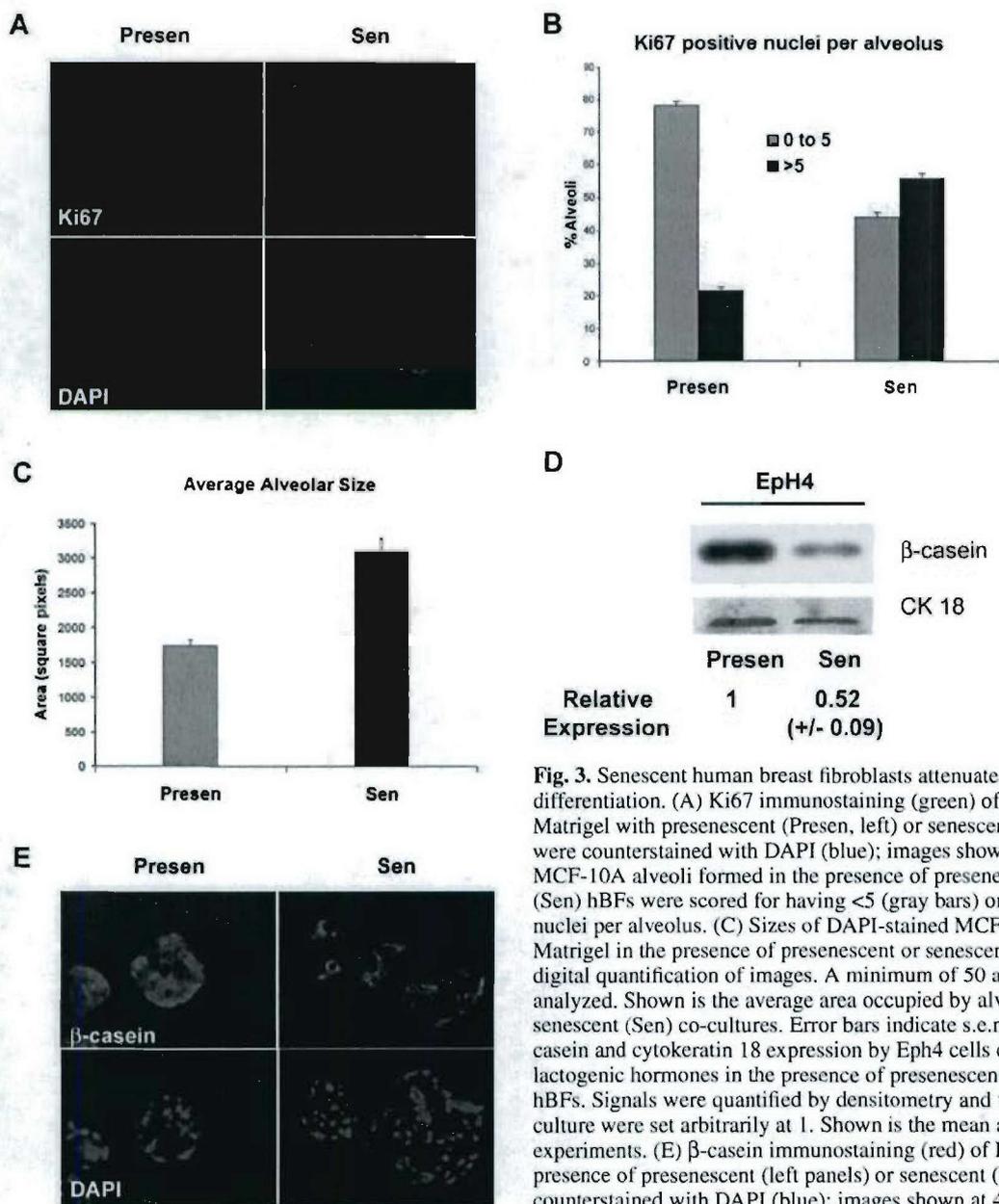


Fig. 3. Senescent human breast fibroblasts attenuate mammary epithelial functional differentiation. (A) Ki67 immunostaining (green) of MCF-10A cells co-cultured on Matrigel with presenescent (Presen, left) or senescent (Sen, right) hBFs. Nuclei were counterstained with DAPI (blue); images shown at 200 \times magnification. (B) MCF-10A alveoli formed in the presence of presenescent (Presen) or senescent (Sen) hBFs were scored for having <5 (gray bars) or >5 (black bars) Ki67-positive nuclei per alveolus. (C) Sizes of DAPI-stained MCF-10A alveoli formed on Matrigel in the presence of presenescent or senescent hBFs were determined by digital quantification of images. A minimum of 50 alveoli per condition was analyzed. Shown is the average area occupied by alveoli in presenescent (Presen) or senescent (Sen) co-cultures. Error bars indicate s.e.m. (D) Western analysis of β -casein and cytokeratin 18 expression by Eph4 cells cultured on Matrigel plus lactogenic hormones in the presence of presenescent (Presen) or senescent (Sen) hBFs. Signals were quantified by densitometry and values from the presenescent culture were set arbitrarily at 1. Shown is the mean and standard deviation from four experiments. (E) β -casein immunostaining (red) of Eph4 alveoli cultured in the presence of presenescent (left panels) or senescent (right panels) hBFs. Nuclei were counterstained with DAPI (blue); images shown at 400 \times magnification.

contrast to their effects on branching morphogenesis (discussed below), the effects of senescent fibroblasts on alveolar differentiation were not due to their elevated secretion of MMPs (Krtolica and Campisi, 2002; Millis et al., 1992). Addition of the general MMP inhibitor GM6001, or a specific MMP-3 inhibitor, failed to reduce the Ki67 labeling of MCF-10A cells, or rescue the lactogenesis defect of Eph4 cells, co-cultured in 3D with senescent hBFs (not shown).

Senescent fibroblasts alter branching morphogenesis of normal mammary epithelial cells

Ductal branching in the mammary gland entails the controlled migration and invasion of epithelial cells through the stromal ECM. Branching morphogenesis is strongly influenced by the stroma, and stromal fibroblasts regulate mammary epithelial branching predominantly through the secretion of soluble factors (Fata et al., 2004; Woodward et al., 1998).

To determine whether senescent fibroblasts and the factors they secrete alter branching differentiation, we established branching assays using primary epithelial organoids and stromal fibroblasts (mBF) from virgin mouse mammary

glands. For these assays, we used mouse mammary fibroblasts (mBFs) in order to create as much as possible a physiologically relevant homotypic system. In addition, we performed these experiments in a 3% oxygen atmosphere. We have shown that the growth arrest of mouse cells in atmospheric (21%) oxygen is due to oxygen toxicity, and that murine cells do not undergo replicative senescence in physiological (e.g. 3%) oxygen concentrations (Parrinello et al., 2003). We therefore induced senescence in mBFs cultured in 3% oxygen by X-irradiation. We confirmed senescence by the cell morphology, %LN (<10%) and SA- β gal expression (not shown).

We carried out branching assays using transwells, which have two chambers separated by a porous membrane that allows an exchange of soluble factors, but not cells. We embedded primary organoids in a 3D collagen gel and plated them in the upper transwell chambers. We plated presenescent or senescent (X-irradiated) mBFs in the lower chambers. We also established control transwells containing epithelial organoids in collagen without fibroblasts in the lower chambers. We maintained all the co-cultures in serum-free medium.

Control cultures lacking fibroblasts showed little or no

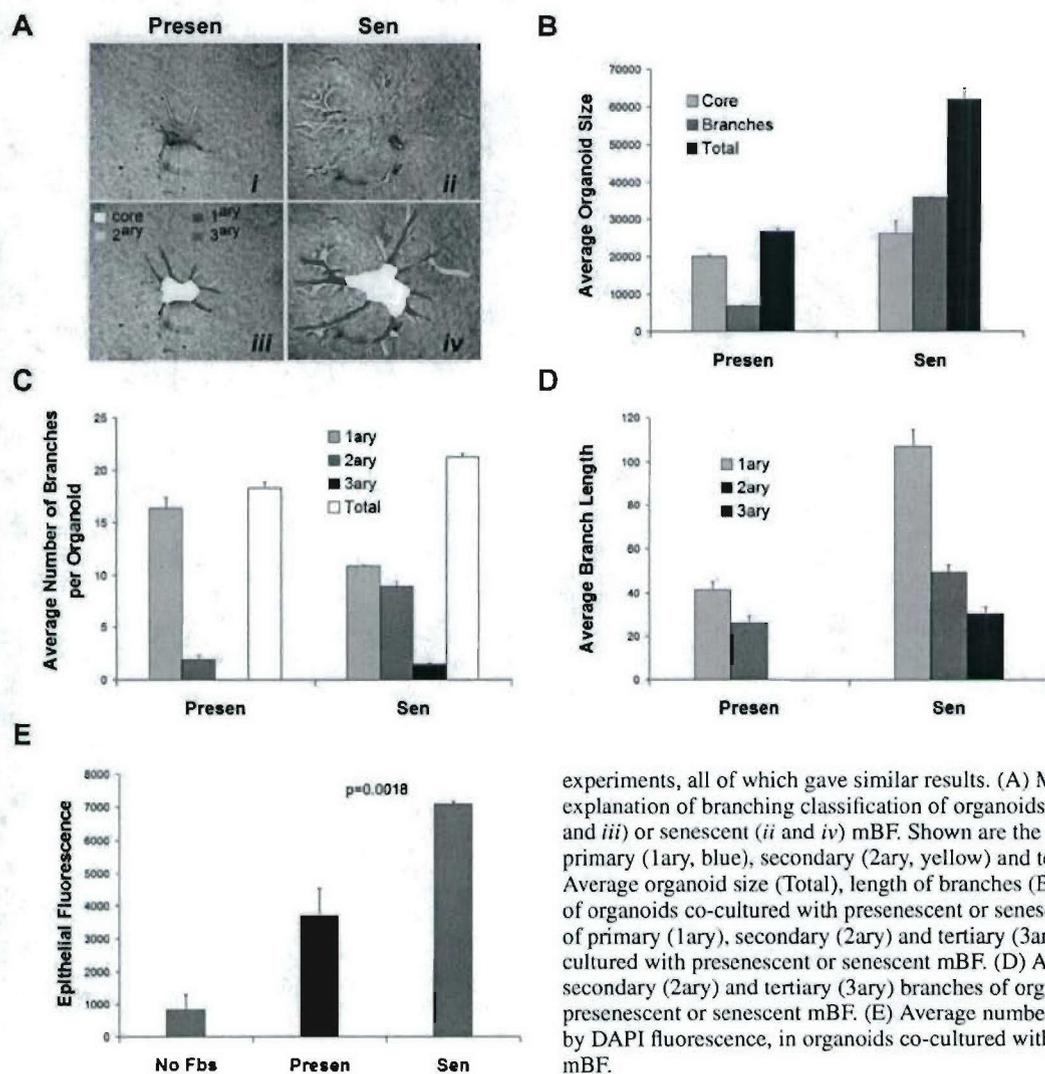


Fig. 4. Senescent mouse fibroblasts stimulate mammary epithelial branching morphogenesis. Primary organoids were embedded in collagen and plated in the upper chambers of transwells. Presenescent (Presen) or senescent (Sen) mBF were plated in the transwell lower chambers. Error bars show s.e.m. of triplicate wells. Shown is one of four

experiments, all of which gave similar results. (A) Morphology and color coded explanation of branching classification of organoids cultured with presenescent (*i* and *iii*) or senescent (*ii* and *iv*) mBF. Shown are the core areas (white), and the primary (1ary, blue), secondary (2ary, yellow) and tertiary (3ary, red) branches. (B) Average organoid size (Total), length of branches (Branches) and core areas (Core) of organoids co-cultured with presenescent or senescent mBF. (C) Average number of primary (1ary), secondary (2ary) and tertiary (3ary) branches of organoids co-cultured with presenescent or senescent mBF. (D) Average length of primary (1ary), secondary (2ary) and tertiary (3ary) branches of organoids co-cultured with presenescent or senescent mBF. (E) Average number of epithelial nuclei, quantified by DAPI fluorescence, in organoids co-cultured with presenescent or senescent mBF.

branching (not shown). However, the presence of fibroblasts stimulated within 2-4 days the formation of projections reminiscent of ductal branching, as reported (Zhang et al.,

2002) (Fig. 4*Ai,ii*). Strikingly, epithelial branching was significantly more pronounced and extensive in co-cultures that contained senescent fibroblasts (Fig. 4*Aii*), relative to those

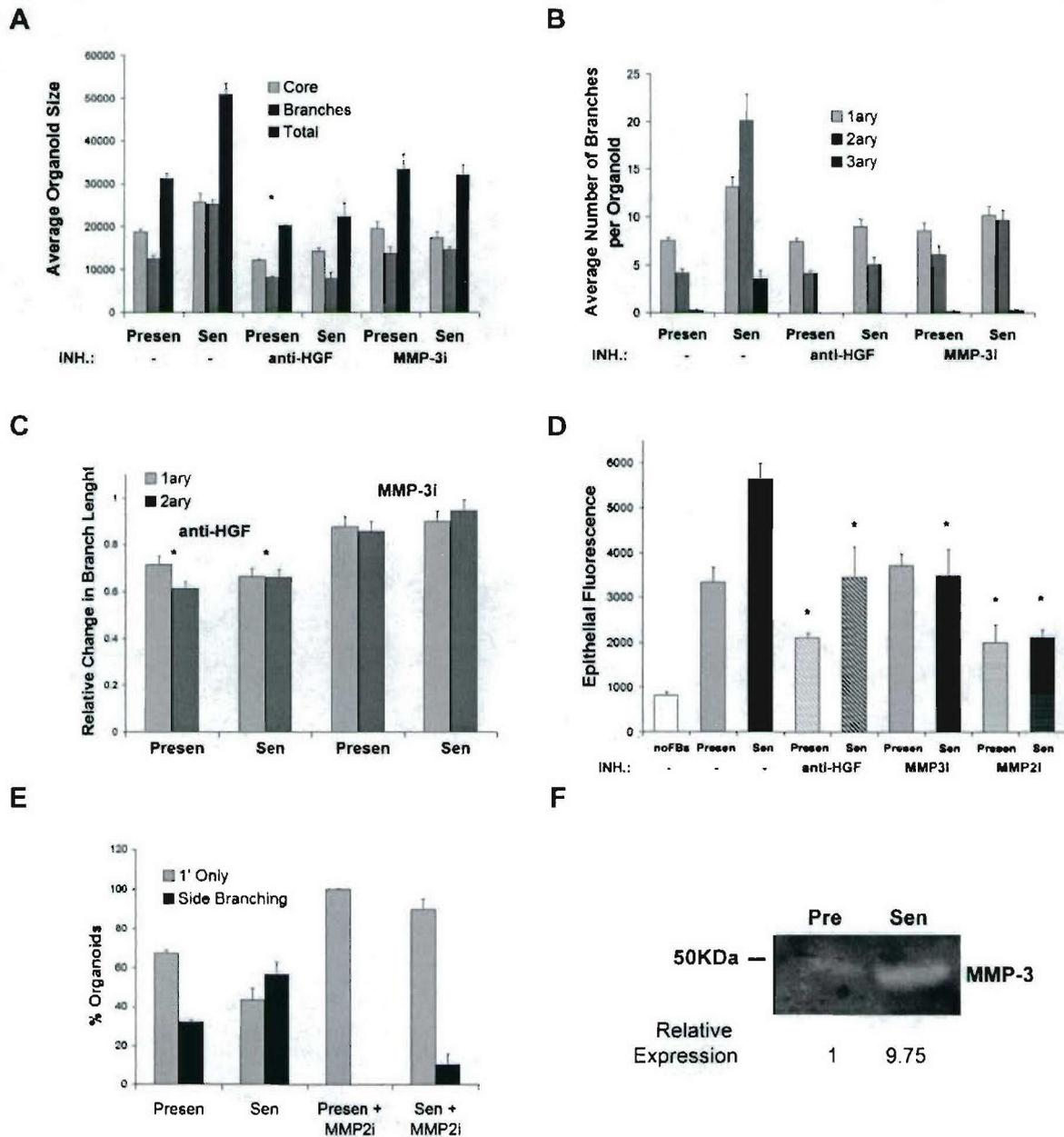


Fig. 5. Senescent fibroblast-produced factors stimulate epithelial branching. Collagen-embedded organoids were cultured in the presence of conditioned medium from presenescent (Presen) or senescent (Sen) mBF. The conditioned medium was either unsupplemented (–) or preincubated with HGF blocking antibody (anti-HGF), an MMP-3 blocking peptide (MMP-3i) or an MMP-2 inhibitor (MMP-2i). Error bars show s.e.m. of triplicate wells. (A) Average size (Total), branch length (Branches) and core area (Core) of organoids cultured with conditioned media lacking or containing an HGF blocking antibody or MMP-3i. (B) Average number of primary (1ary), secondary (2ary) and tertiary (3ary) branches in the organoids analyzed in A. (C) Organoids in A were analyzed for average primary and secondary branch lengths in the presence of HGF blocking antibody or MMP-3i, and expressed as change relative to presenescent or senescent-derived conditioned medium lacking antibody or inhibitor. The asterisks indicate a statistically significant reduction in branch length by HGF neutralization, compared to medium lacking HGF antibody, as determined by a Student's *t*-test. Branching length in presenescent and senescent conditioned media was affected similarly by HGF neutralization. (D) Average number of epithelial cells per organoid, quantified by DAPI fluorescence of nuclei. Asterisks indicate a statistically significant change in proliferation, as determined by a Student's *t*-test. (E) Percentage of organoids having primary (1' Only) or higher level (secondary and tertiary; Side Branching) branches in the absence or presence of MMP-2i. (F) Casein zymography of conditioned medium from presenescent and senescent mBF. A 50 kDa marker and MMP-3 are indicated. Densitometry was performed on the reverse image to determine the relative expression; the signal from presenescent conditioned medium was set arbitrarily at 1.

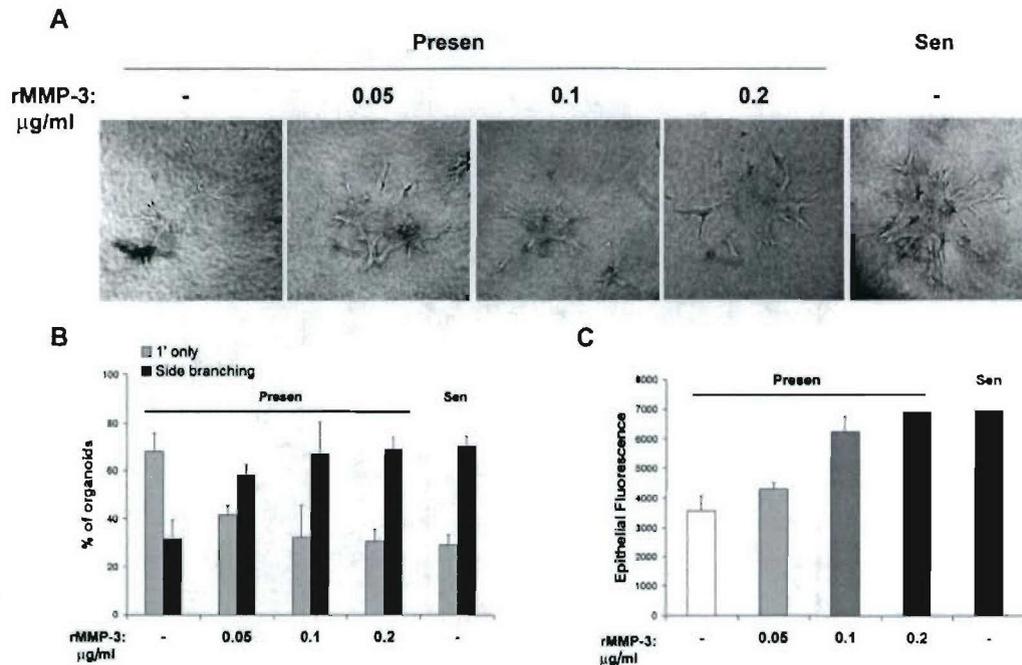


Fig. 6. Role of MMP-3 in branching stimulated by senescent fibroblasts. (A) Morphology of representative collagen-embedded organoids after culture in the presence of conditioned medium from presenescent (Presen) mBF containing the indicated amounts of recombinant MMP-3. An organoid cultured with conditioned medium from senescent (Sen) (X-irradiated) mBF is shown for comparison. (B) Percentage of organoids that undergo primary (1' only) or higher level (secondary and tertiary; Side branching) branching in the presence of presenescent mBF-conditioned medium containing the indicated concentrations of recombinant MMP-3. The effect of conditioned medium from senescent (Sen) mBF is shown for comparison. (C) Proliferation of organoids, determined by DAPI fluorescence, in the presence of presenescent mBF-conditioned medium containing the indicated concentrations of recombinant MMP-3. The effect of conditioned medium from senescent (Sen) mBF is shown for comparison.

that contained presenescent fibroblasts (Fig 3Ai). Specifically, organoids cultured with senescent mBFs were larger and had more branches (Fig. 4Aiii,iv,B). They also had significantly more secondary and tertiary branching (Fig. 4C) and longer projections (branch length; Fig. 4D), as determined by digital quantification of the images (Fig. 4B-D). In addition, senescent mBFs stimulated the proliferation of the organoid epithelial cells, as determined by quantification of DAPI-stained epithelial nuclei (Krtolica et al., 2002) (Fig. 4E) and MTS assays for viable cells (Cory et al., 1991) (not shown).

We conclude that senescent fibroblasts can alter the functional and morphological differentiation of mammary epithelial cells. These alterations probably occur in part because senescent cells stimulate epithelial cell growth, and additionally because senescent cells stimulate the migration and invasion (branching) of the epithelial cells through a collagen matrix.

Senescent fibroblasts stimulate branching morphogenesis via MMP-3

Several hormones and growth factors are important for branching morphogenesis in the mammary gland, but hepatocyte growth factor (HGF), MMP-2 and MMP-3 are of particular interest because they are expressed by the stroma (Fata et al., 2004; Woodward et al., 1998). To determine whether these stromal factors were responsible for the branching morphogenesis stimulated by senescent fibroblasts,

we performed branching assays in the presence of blocking antibodies or specific inhibitors, substituting fibroblast-conditioned medium collected over a 48-hour interval for fibroblasts. This substitution allowed us to more accurately normalize the cultures for fibroblast cell number, and more effectively block the activity of HGF, MMP-2 and MMP-3 by preincubating the conditioned media with antibodies or inhibitors.

To assess the contribution of HGF, we first determined that presenescent and senescent fibroblasts expressed HGF to similar extents, as measured by quantitative real-time polymerase chain reactions (RT-PCR; not shown). Consistent with this finding and the importance of HGF for branching morphogenesis, blocking antibody against HGF reduced both the size of the organoid core and total extent of branching (Fig. 5A). The HGF-blocking antibody reduced organoid size and branching regardless of whether the cultures contained conditioned medium from presenescent or senescent fibroblasts (Fig. 5A). In both cases, HGF neutralization suppressed the formation of tertiary branches (Fig. 5B), the average length of primary and secondary branches (Fig. 5C) and the proliferation of cells in the organoids (Fig. 5D). The HGF antibody was more active in suppressing the number of primary and secondary branches stimulated by senescent, compared to presenescent, conditioned medium (Fig. 5B). Taken together, these results indicate that HGF is necessary for organoid branching, as reported previously (Simian et al., 2001; Zhang et al., 2002). However, although HGF appeared

to contribute to the greater number of primary and secondary branches stimulated by senescent fibroblasts, HGF did not appear to be responsible for the difference between presenescent and senescent fibroblasts in their ability to stimulate tertiary branching or cell proliferation. In contrast to HGF-blocking antibodies, blocking antibodies against epidermal growth factor (EGF) or insulin-like growth factor-1 (IGF-1) had no effect on organoid size and branching (not shown). Likewise, a specific inhibitor of MMP-2 (MMP-2i) reduced organoid cell proliferation (Fig. 5D), and visual inspection of the organoids indicated that it suppressed secondary and tertiary (side) branching (Fig. 5E), as reported by Wiseman et al. (Wiseman et al., 2003). However, the inhibition was not selective to the stimulation caused by senescent fibroblasts. This result suggests that MMP-2, like HGF, cannot be solely responsible for the stimulation of branching by senescent fibroblasts.

In contrast to the effects of HGF and MMP-2 inhibition, MMP-3 inhibition selectively suppressed the stimulation of branching by senescent fibroblasts. MMP-3 expression is known to be upregulated in senescent human fibroblasts (Millis et al., 1992). We confirmed that senescent mBFs also overexpress MMP-3 mRNA (not shown), and determined by zymography that they secrete 10-fold more MMP-3 than presenescent mBFs (Fig. 5F). A peptide inhibitor of MMP-3 (MMP-3i) had little effect on organoid branching (Fig. 5A,B) or cell proliferation (Fig. 5D) in cultures containing conditioned medium from presenescent fibroblasts. However, the inhibitor sharply reduced the number of secondary and tertiary branches, and the amount of cell proliferation, in cultures containing conditioned medium from senescent fibroblasts (Fig. 5A,B,D). Moreover, the MMP-3i brought the branching and proliferation caused by senescent fibroblasts to the level caused by presenescent fibroblasts (Fig. 5B,D). Likewise, expression of an MMP-3 shRNA that partially reduced MMP-3 expression in senescent mBFs, also partially reduced the ability of conditioned medium from these cells to stimulate secondary and tertiary branching (not shown). Together, these results suggest that MMP-3 may be a major contributing factor to the effects of senescent fibroblasts on mammary epithelial branching differentiation. Consistent with this idea, addition of recombinant MMP-3 to conditioned medium produced by presenescent fibroblasts stimulated branching, including extensive side (secondary and tertiary) branching, in a dose-dependent manner, eventually reaching the level caused by senescent fibroblasts (Fig. 6A,B). Recombinant MMP-3 also stimulated the proliferation of epithelial cells in the organoids, also in a dose-dependent manner and also eventually reaching the level of stimulation caused by senescent fibroblasts (Fig. 6C).

These findings indicate that senescent fibroblasts alter branching morphogenesis in primary mammary organoids, in large measure because of their elevated secretion of MMP-3, which acts together with HGF and MMP-2.

Discussion

The senescence response is very probably a tumor suppressive mechanism that evolved to arrest the growth of cells at risk for malignant transformation (Campisi, 2003). In addition, increasing evidence suggests that senescent cells may

contribute to aging phenotypes. Consistent with this view, senescent cells have been shown to accumulate with age in rodent and human tissues (reviewed by Krtolica and Campisi, 2002; Campisi, 2003). They have also been identified at sites of certain age-related pathologies, including atherosclerotic plaques (Vasile et al., 2001), hyperplastic lesions in the prostate (Choi et al., 2000) and preneoplastic lesions in the liver (Paradis et al., 2001). More direct evidence that senescent cells can have biological effects derives from studies showing that senescent fibroblasts can stimulate the proliferation of premalignant epithelial cells in culture and the tumorigenic conversion of such cells in vivo (Krtolica et al., 2001). Together, these findings suggest that the senescence response may be an example of evolutionary antagonistic pleiotropy. According to this idea, the senescence response may prevent early life cancers by irreversibly arresting the proliferation of potential cancer cells. Later in life, however, as senescent cells accumulate, they may disrupt normal tissue structure and/or function. As such, senescent cells may then contribute to late life cancers by creating a tissue microenvironment that is permissive for malignant progression of cells that harbor oncogenic mutations.

Our earlier studies showed that senescent human fibroblasts stimulate the growth of premalignant (immortal but not tumorigenic) mouse and human epithelial cells in two-dimensional co-culture assays. Here, we show that even in 3D co-culture assays, which more accurately approximate physiological conditions, senescent fibroblasts stimulate the proliferation of premalignant mouse and human epithelial cells. Strikingly, however, although senescent fibroblasts did not stimulate normal epithelial cell growth in two-dimensional co-cultures (Krtolica et al., 2001), we show here that they stimulate the growth of normal epithelial cells in more physiological 3D assays. This growth stimulation most likely contributes to the ability of senescent fibroblasts to alter the differentiation of the epithelial cells. However, it is unlikely that stimulated proliferation per se can explain the increase in branching differentiation, which requires that epithelial cells invade and migrate through a surrounding collagen matrix.

The ability of epithelial cells to migrate and invade the basement membrane and stroma are stringently controlled in normal tissues, a control that is lost upon malignant transformation. Our results suggest that senescent stromal cells can relax the constraints on epithelial cell proliferation, migration and invasiveness that are normally imposed by the tissue microenvironment. Of particular importance and relevance to the link between aging and cancer, our results suggest that senescent stromal cells can stimulate the growth and promote the acquisition of invasive and migratory phenotypes by normal or premalignant epithelial cells. Consistent with this idea, senescent fibroblasts that were co-injected with premalignant SCp2 mouse mammary epithelial cells caused a loss of epithelial differentiation, acquisition of invasiveness, possibly by an EMT, and full malignant transformation. Moreover, senescent fibroblasts, when co-cultured in 3D with basement membrane components (Matrigel) and immortal mouse or human mammary epithelial cells, disrupted alveolar morphogenesis and functional differentiation. These effects were probably not due to the elevated expression of MMP-3 or other MMPs by senescent fibroblasts because the MMP inhibitors did not prevent the

inhibition of alveolar morphogenesis or lactogenesis. Likewise, the stimulation of cell proliferation during alveolar and functional differentiation was not suppressed by MMP-3 inhibition (not shown). These results are consistent with previous findings in two-dimensional co-cultures, where the stimulation of epithelial growth induced by senescent fibroblasts could not be inhibited by MMP inhibition (our unpublished data). Thus, at least some of the effects of senescent fibroblasts are probably caused by more complex stromal-epithelial interactions, involving both soluble and insoluble factors, as reported previously (Krtolica et al., 2001). Whatever the cause, our findings demonstrate that senescent stromal cells can compromise the function of mammary, and possibly other, epithelial cells.

In contrast to their effects on epithelial cell growth and alveolar morphogenesis, we found that senescent fibroblasts stimulated branching morphogenesis by normal mammary epithelial organoids primarily because of their secretion of MMP-3. Branching morphogenesis entails migration and invasion through collagen. The senescence response markedly increases MMP-3 expression by both mouse and human fibroblasts, although the inducer(s) of MMP-3 in senescent cells is not known. HGF has been shown to induce MMP-3 expression in keratinocytes (Dunsmore et al., 1996) and thus is a potential stimulator of MMP-3 expression in senescent fibroblasts. Arguing against this possibility, HGF expression was similar in presenescent and senescent mouse fibroblasts, as determined by quantitative RT-PCR, although we cannot rule out the possibility that expression or activation of the HGF receptor, c-Met, might be elevated in senescent cells. In addition, we assayed MMP-3 in conditioned media from senescent mBF cultures that had been pre-treated with HGF blocking antibodies. Casein zymography showed no difference in MMP-3 levels between mock-treated and HGF antibody-treated cultures (not shown), suggesting that MMP-3 is induced by HGF-independent mechanisms in senescent fibroblasts.

MMP-3 is crucial for branching morphogenesis, especially secondary and tertiary branching, in the differentiating mammary gland (Simian et al., 2001; Wiseman et al., 2003). By inhibiting MMP-3 in the conditioned medium produced by senescent fibroblasts, and supplementing presenescent fibroblast-conditioned medium with recombinant MMP-3, we identified MMP-3 as a prime candidate for mediating the effects of senescent fibroblasts on branching morphogenesis. What might be the significance of the increased MMP-3 secretion by senescent stromal cells *in vivo*? Ectopic high-level expression of MMP-3 in the mammary gland markedly increases the incidence of epithelial breast cancers in mice, presumably because it disrupts the normal tissue architecture and creates a tissue environment that promotes the malignant progression of initiated epithelial cells (Sternlicht et al., 1999). The MMP-3 produced by senescent fibroblasts may likewise promote a tissue structure and microenvironment that stimulates the progression of resident premalignant epithelial cells. Moreover, our results indicate that the MMP-3 produced by senescent fibroblasts also stimulates epithelial cell proliferation, which in turn can favor oncogenesis by fueling, and subsequently fixing, mutations. Finally, even in the absence of nearby premalignant cells, factors produced by senescent stromal cells may facilitate the development of

hyperproliferative lesions in the breast and other epithelial organs, which increase with age.

Our finding that senescent fibroblasts affect both the morphological organization and function of mammary epithelial cells may also be relevant to aging phenotypes. Loss of tissue structure and function are hallmarks of aging (Brelinska et al., 2003; Kirkland et al., 2002). In this regard, the mammary gland may be a model for other tissues, which, when structure or function are altered, can compromise organismal health and fitness. For example, the presence of senescent dermal fibroblasts in cell culture models of skin promoted subdermal blistering and epidermal fragility, which can occur in aged skin (Funk et al., 2000). Likewise, senescent endothelial cells, which secrete high levels of the inflammatory cytokine IL-1 (Maier et al., 1990), have been identified in, and proposed to initiate, atherosclerotic lesions in human aorta (Vasile et al., 2001). Thus, in addition to contributing to late life malignancies, senescent cells might contribute to a variety of aging phenotypes and non-neoplastic age-related pathologies.

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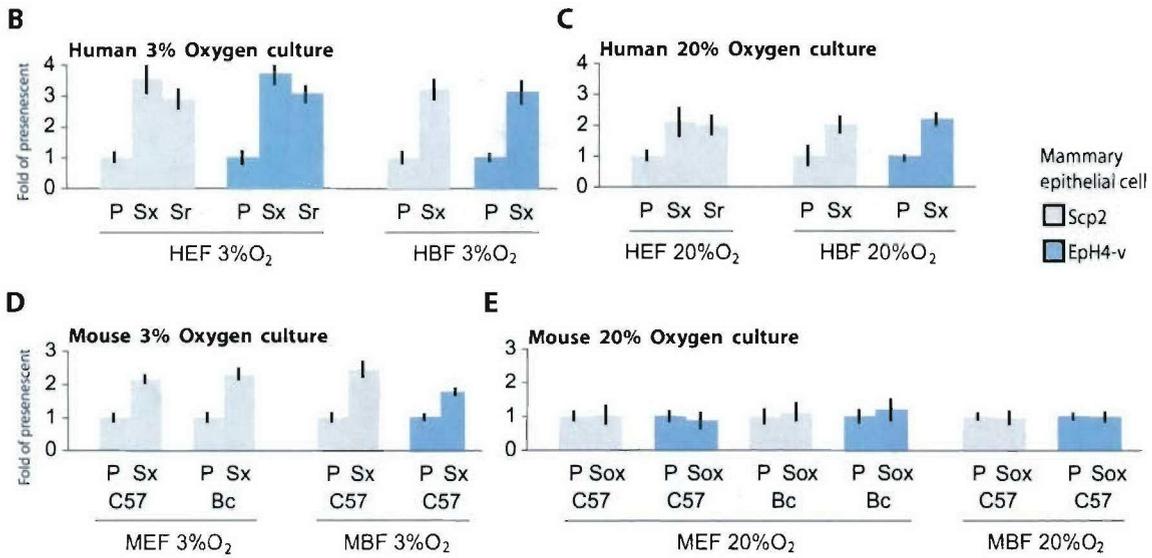
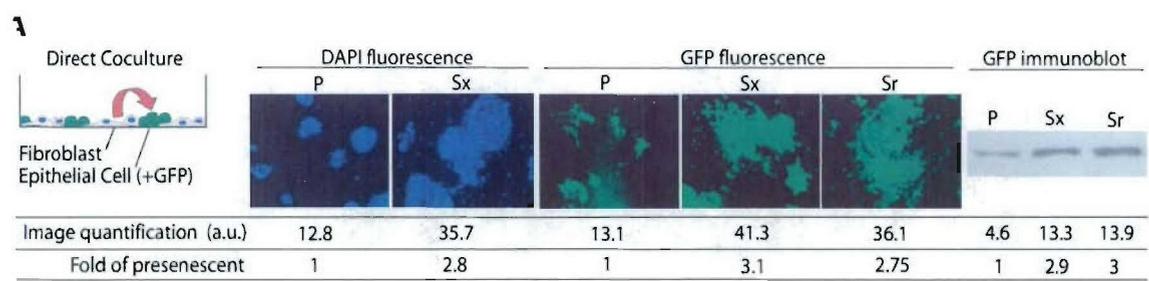


FIGURE 1

LEGEND :

Stromal fibroblast used as feeder-layer for co-culture with epithelial cells :

P = pre-senescent

Sx = senesced by X-irradiation

Sr = senesced by replicative exhaustion

Sox = senesced by hyper-oxidative stress

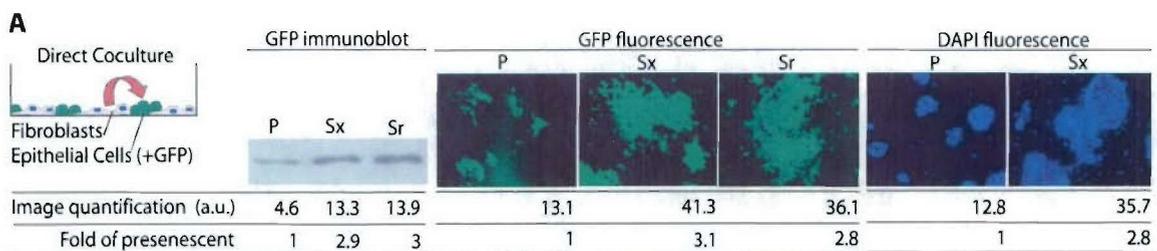
HEF / MEF = Human / Mouse Embryonic Fibroblast

HBF / MBF = Human / Mouse adult Breast Fibroblast

Mammary epithelial cell used :

□ **Scp2** (+/- GFP)

■ **EpH4-v** (+/- GFP)



Inducing a human-like senescent phenotype in mouse fibroblasts

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Mammalian cells can respond to stress or damage by undergoing a permanent cell cycle arrest termed cellular senescence. The senescence response suppresses the development of cancer, but the altered cellular functions that accompany this response may contribute to aging. We have shown that the phenotype of mouse fibroblasts that arrest growth when cultured in 20% oxygen does not resemble that of senescent human fibroblasts, and that mouse fibroblasts do not arrest growth when cultured in a physiological (3%) oxygen environment. We now report on conditions under which mouse fibroblasts acquire a phenotype similar to that of senescent human fibroblasts. Towards this end we characterized the secretory phenotype of human and mouse fibroblasts and studied the effects of senescent stroma on epithelial cell growth and tumorigenesis.

In contrast to mouse fibroblasts that spontaneously arrest growth in 20% oxygen, mouse fibroblasts cultured in 3% oxygen and subsequently X-irradiated behaved similarly to human fibroblasts made senescent by replicative exhaustion or X-irradiation. Similarities included the lack of c-fos response to serum and upregulated expression of PAI-1, TIMPs, and MMPs. Antibody array experiments showed that X-irradiated mouse fibroblasts cultured in 3% oxygen secreted a profile of pro-carcinogenic and inflammatory molecules similar to those secreted by senescent human fibroblasts cultured in either 3% or 20% oxygen. The observed mouse and human senescent cytokine profiles resembled those implicated in the pathophysiology of aging *in vivo*. Moreover, X-irradiated mouse fibroblasts cultured in 3% oxygen promoted the hyperproliferation of premalignant mouse epithelial cells, similar to the effects we observed of senescent human fibroblasts. *In vivo* tumorigenesis studies are currently in progress. Our data provide a basis for creating an *in vivo* mouse model of human cellular senescence, and studying effects of senescent stromal cells on aging and cancer *in vivo*.

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Senescent stromal cells alter mammary gland differentiation

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Cellular senescence is a tumor suppressive mechanism that permanently arrests cells at risk for malignant transformation. We have previously shown that senescent cells promote tumorigenesis suggesting the senescence response is antagonistically pleiotropic. This is in large extent due to the secretion of matrix-degrading and growth stimulatory factors by senescent stromal cells. Here, we show some of the characteristics of this phenotype and its effects on mammary gland differentiation and tumorigenesis. Premalignant mammary epithelial cells exposed to senescent human fibroblasts in mice irreversibly lose differentiated properties, become invasive and undergo full malignant transformation. Moreover, using mouse or human fibroblasts and non-malignant breast epithelial cells in culture, we demonstrate that senescent fibroblasts disrupt normal epithelial alveolar morphogenesis and functional differentiation, suggesting that senescent stromal cells can contribute to loss of normal tissue function. In addition, senescent fibroblasts stimulated inappropriate branching morphogenesis, suggesting that senescent stromal cells can also stimulate hyperplastic changes in normal tissues. We identify MMP-3 as a major factor responsible for the effects of senescent fibroblasts on branching morphogenesis. Our findings support the idea that senescent cells contribute to age-related pathology, including cancer. In addition, we describe a new property of senescent fibroblasts - the ability to alter epithelial differentiation - that might explain the loss of tissue function and organization that is a hallmark of aging.

INDUCING A HUMAN-LIKE SENESCENT PHENOTYPE IN MOUSE FIBROBLAST PROMOTES MAMMARY EPITHELIAL CELL PROLIFERATION AND TUMORIGENESIS: A MOUSE MODEL FOR HUMAN AGING.

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Mammalian cells can respond to stress or damage by undergoing a permanent cell cycle arrest termed cellular senescence. The senescence response suppresses the development of cancer, but the altered cellular functions that accompany this response may contribute to aging. We have shown that the phenotype of mouse fibroblasts that arrest growth when cultured in 20% oxygen does not resemble that of senescent human fibroblasts, and that mouse fibroblasts do not arrest growth when cultured in a physiological (3%) oxygen environment. We now report on conditions under which mouse fibroblasts acquire a phenotype similar to that of senescent human fibroblasts.

Towards this end we characterized (*i*) the secretory phenotype of human and mouse mammary and embryonic fibroblasts and (*ii*) studied the effects of senescent stroma on their tissue microenvironment. In particular, mammary epithelial cell growth *in vitro* and tumorigenesis *in vivo*.

In contrast to mouse fibroblasts that spontaneously arrest growth in 20% oxygen, mouse fibroblasts cultured in 3% oxygen and subsequently X-irradiated behaved similarly to human fibroblasts made senescent by replicative exhaustion or X-irradiation. Similarities included the lack of c-fos response to serum and upregulated expression of PAI-1, TIMPs, and MMPs. Antibody array experiments showed that X-irradiated mouse fibroblasts cultured in 3% oxygen secreted a profile of pro-carcinogenic and inflammatory molecules similar to those secreted by senescent human fibroblasts cultured in either 3% or 20% oxygen. The observed mouse and human senescent cytokine profiles resembled those implicated in the pathophysiology of aging *in vivo*. Moreover, X-irradiated mouse fibroblasts cultured in 3% oxygen promoted the hyperproliferation of premalignant mouse epithelial cells, similar to the effects we observed with senescent human fibroblasts. As previously observed with senescent human fibroblast, *in vivo* tumorigenesis studies showed that only X-irradiated mouse fibroblasts cultured under 3% oxygen conditions promote pre-neoplastic mammary epithelial cell tumorigenesis.

We have been able to establish *in vitro* conditions for mouse fibroblasts to mimic the physiological effects of human stromal aging. We defined at the molecular level how fibroblasts can affect their microenvironment, in particular in models of human and mouse mammary gland carcinogenesis. Our data provide a basis for creating an *in vivo* mouse model of human cellular senescence, and studying effects of senescent stromal cells on aging and cancer *in vivo*. Once such a model is established, it will have much improved applications for exploring the effects of aging on breast cancer and for developing new treatments aimed to counteract senescent changes in the microenvironment.

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