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PRINCIPAL INVESTIGATOR: Ana Krtolica, Ph.D.
J. Campisi, Ph.D.

CONTRACTING ORGANIZATION: Lawrence Berkeley National Labortory
One Cyclotron Road, MS 70A-1118
Berkeley, California 94720

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Ana Krtolica, Ph.D.
J. Campisi, Ph.D.

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Cancer incidence rises exponentially with age. This project tests the hypothesis that replicative senescence of stromal fibroblasts contributes to the age-dependent increase in breast cancer by creating a more permissive environment for the expression of malignant phenotypes by breast epithelial cells.

Here, we report that both mouse and human immortal pre-malignant breast epithelial cell lines show increased proliferation in the presence of senescent, compared to presenescent, human fibroblasts. At least part of this senescent fibroblast-derived mitotic activity is due to secreted insoluble matrix components. The effect of senescent fibroblasts on breast epithelial cell proliferation is observable even when they are present as only 10% of a mixed population of presenescent and senescent fibroblasts. One very important implication of this finding is that the accumulation of even a few senescent cells in the aging organism may stimulate unconstrained epithelial growth and thus contribute to malignant transformation of the surrounding breast epithelium. Taken together these results support our hypothesis that replicative senescence of stromal fibroblasts may contribute to the development of malignant properties of breast epithelial cells.

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Ana [illegible] 8/30/99

PI - Signature Date

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Introduction

Age is the main risk factor for the majority of human cancers, including breast cancer. The goal of this project is to investigate whether cellular senescence contributes to this age-related rise in cancer frequency. Specifically, we want to test the hypothesis that replicative senescence of stromal fibroblasts alters the environment of breast epithelial cells such that it becomes permissive for the expression of malignant phenotypes. With this in mind, we created cell culture systems that allowed us to explore the influence of senescent fibroblasts on breast epithelial cell behavior. In particular, we developed methods to follow changes in those breast epithelial cell characteristics that may contribute to development of malignant phenotypes: cell proliferation, differentiation and invasiveness. Using this experimental system we hope to contribute to understanding the mechanism by which the aging of stromal cells creates a microenvironment that can contribute to breast cancer pathogenesis.

Body of the Annual Summary

Senescent and presenescent fibroblasts can influence breast epithelial cells in multiple ways:

- through secretion of soluble factors (independent or dependent on breast cell stimuli)
- by releasing an insoluble extracellular matrix
- by degradation of the extracellular matrix surrounding breast cells (e.g. basement membrane)
- by cell-cell interactions

We have designed multiple strategies to evaluate either independently, or in combination, each of these possible fibroblast influences on the breast epithelial cells. In our experiments, we have used human breast epithelial cell lines with different degrees of malignity: S1 was derived from a benign tumor (obtained from Mina Bissell's laboratory), MD MBA 453 was from breast carcinoma, and ErbB2-infected B5 cells are anchorage independent (from laboratory of Martha Stamper and Paul Yaswen). In addition, we have used SCp2 non-tumorigenic mouse mammary cells, which are capable of differentiation (obtained from Pierre Desprez). Presenescent and senescent fibroblasts were from human fetal lung, WI38, and normal human breast, derived from a 16-year-old patient.

I designed experimental protocols to evaluate the above-mentioned types of fibroblast-epithelial cell interactions and results that have been obtained so far are briefly described bellow.

1. *Fibroblasts' Conditioned Medium.* This approach tests effects of the soluble factors released by fibroblasts. Presenescent and senescent fibroblast conditioned media were concentrated, dialyzed and concentrated, normalized to the number of cells from which they were derived. Breast epithelial cells were grown in the presence of this conditioned medium and their proliferation and morphology were assessed. This approach gave inconsistent results probably due to the degradation and/or loss of

active components in the media during the preparation process. Further efforts were directed to other strategies.

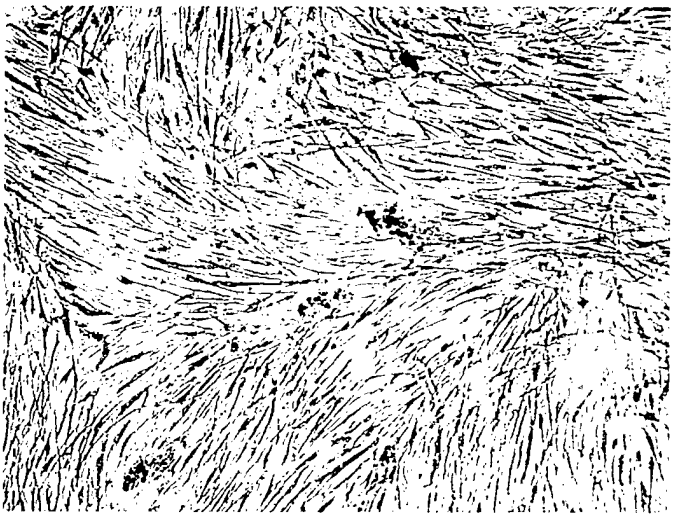
2. *Fibroblast Extracellular Matrices*. In order to determine influence of insoluble matrix proteins produced by fibroblasts, presenescent and senescent WI38 fibroblasts were plated and left to deposit matrix for 2-3 days under serum-free conditions. Fibroblasts were removed by EDTA treatment to avoid protein degradation by trypsin. Immunocytochemical staining showed that fibronectin deposits were not affected by EDTA treatment. Scp2 or S1 cells were subsequently plated on the matrix. Both breast epithelial cell types showed 50% greater proliferation on matrices produced by senescent cells, relative to presenescent fibroblasts.

3. *Breast Cells Grown on Fibroblast Monolayers*. Unlike previous approaches, this co-culture system allows for direct cell-cell interactions, as well as cross-talk between cells (i.e. breast cells can send signal to fibroblasts via soluble factor or through direct cell-cell interaction, which can then induce fibroblasts to secrete factors). WI 38 fibroblasts, from presenescent and senescent cultures were grown 1-3 days at two different densities and then rinsed with serum-free medium. Breast cells were dispersed on top of the fibroblasts. S1 and SCp2 cells made up to 3 times larger colonies on the senescent, than on presenescent fibroblasts (see Figure 1). This enhanced proliferation was roughly independent of the total number of fibroblasts, but dependent on the proportion of senescent versus presenescent fibroblasts (see bellow).

4. *Breast Cells Grown in Matrigel in Upper Chamber of the Transwell with Fibroblasts in the Lower Chamber*. In this experimental system interaction between breast cells and fibroblasts is possible only through soluble factors. Presenescent and senescent fibroblasts were plated in the lower wells of the Transwell chamber and grown for 1-2 days in serum-free conditions. Subsequently, breast cells were resuspended in growth factor–depleted Matrigel and plated in the top well with the porous membrane. There was no significant difference in morphology or colony size for S1, MB MDA 453 and ErbB2-infected B5 cells when presenescent or senescent fibroblasts were in the lower chamber. These results may indicate that the effects on breast epithelial proliferation observed with senescent fibroblasts are due to non-

S1 cells

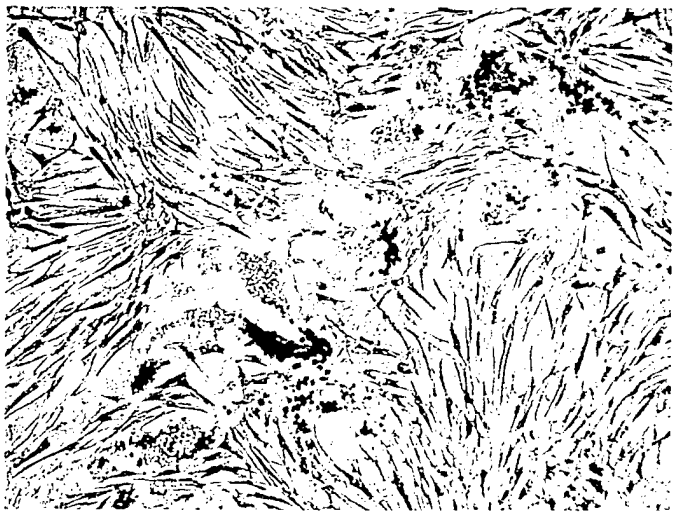
Pre-senescent



Senescent

SCp2 cells

Pre-senescent



Senescent

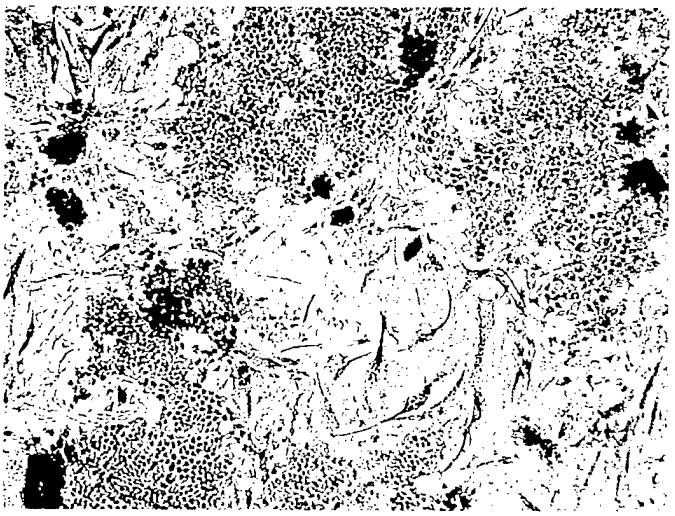


Figure 1

soluble factors. Alternatively, it is reasonable to assume that under *in vivo* conditions growth factors produced by senescent fibroblasts act on adjacent epithelial cells, and that the distance between two cell populations in this experiment far exceeds the distance required for effective concentration for certain secreted factors. We are planning new experimental approaches to test this possibility.

5. *Breast Cells Grown in Matrigel in Upper Chamber of the Transwell with Fibroblasts in the Lower Chamber – Invasion Assay.* This experimental protocol is very similar to 4, except that the cells are plated on the top of the Matrigel and the layer of matrix is much thinner (10μL vs. 300μL/well). Cell invasiveness is quantified by counting the number of cells penetrating the matrix and filter pores to reach the underside of the membrane. There was no significant difference in MB MDA 453 invasiveness in the presence of senescent relative to presenescent breast fibroblasts, when normalized to fibroblast number. S1 cells show very low invasiveness under all conditions for up to 72 hours.

Since the experimental setup with breast epithelial cells grown on top of fibroblasts (discussed under 3) gave the most encouraging results, we used this approach to ask whether senescent fibroblasts stimulated epithelial cell growth in the presence of presenescent fibroblasts. In addition, we wanted to evaluate how many senescent fibroblasts are necessary to stimulate the proliferation of breast epithelial. Presenescent and senescent fibroblasts were mixed at different ratios, plated, left to attach overnight, and washed with serum-free medium. SCp2 breast epithelial cells were plated on top of the fibroblasts and their proliferation was evaluated after 5 to 7 days. A stimulatory effect of senescent fibroblasts on SCp2 colony size was detectable even in the presence of only 10% senescent fibroblasts. This effect increased with an increasing proportion of senescent fibroblasts, with the maximal effect occurring with 100% senescent fibroblasts (see Figure 2; above each picture is the ratio of the number of presenescent and senescent fibroblasts plated). Quantitation from preliminary experiments showed 30% increase in epithelial cell number in the presence of 10% senescent fibroblasts compared to the number of epithelial cells on presenescent fibroblasts alone. We are currently repeating these experiments to obtain more rigorous quantitation.

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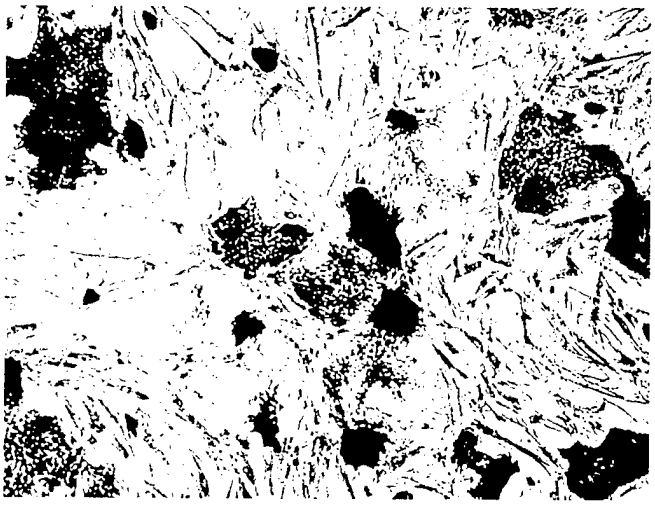

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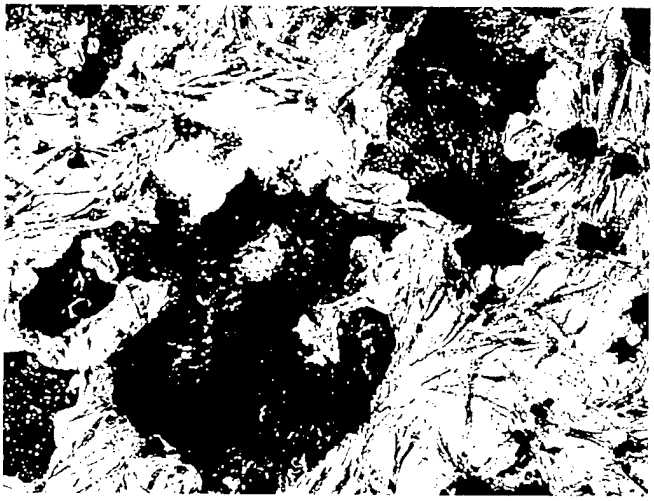

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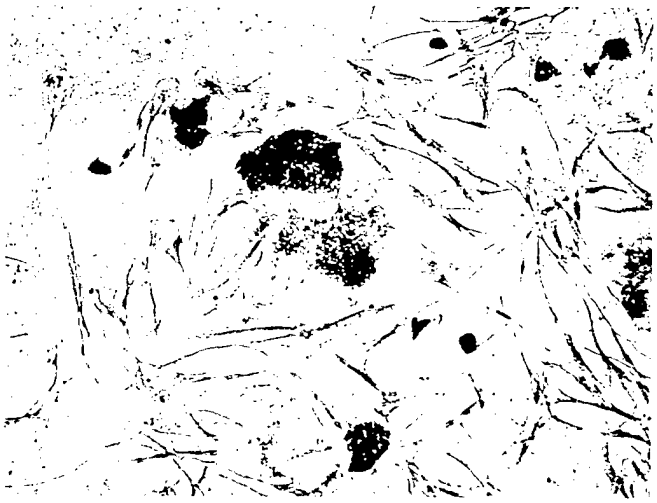

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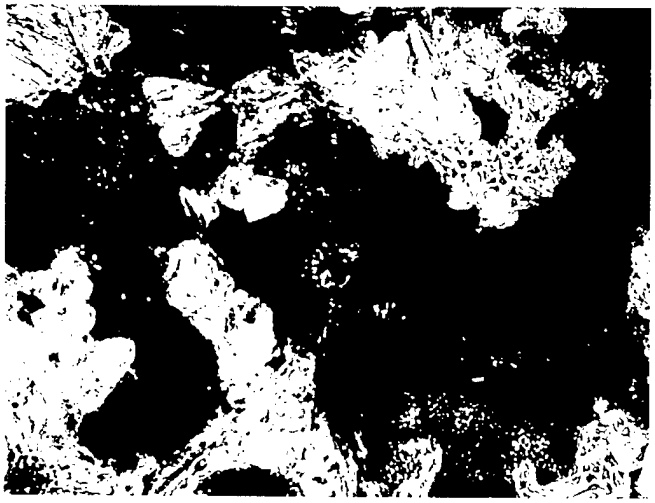

Figure 2

In summary, both mouse and human immortal pre-malignant breast epithelial cell lines show increased proliferation in the presence of senescent relative to presenescent human fibroblasts. At least part of this senescent fibroblast-derived mitotic activity was due to secreted insoluble matrix components. The effect of senescent fibroblasts on breast epithelial cell proliferation is detectable if pre- and senescent fibroblast populations were mixed, even when the percentage of senescent cells was as low as 10%. One very important implication of this finding is that the accumulation of even a few senescent cells in the aging organism may stimulate unconstrained epithelial growth and thus contribute to malignant transformation of the surrounding breast epithelium. Taken together, these results give support to our hypothesis that replicative senescence of stromal fibroblasts may contribute to the development of malignant properties of breast epithelial cells.

List of Key Research Accomplishments

1. Senescent human fibroblasts stimulate a breast epithelial cell proliferation relative to presenescent fibroblasts.
2. Senescent fibroblast-derived extracellular matrix contributed to the increase in breast epithelial cell proliferation.
3. The effect of senescent fibroblasts on breast epithelial cell proliferation was observable even in a mixed pre- and senescent fibroblast population, and was still detectable with only 10% senescent fibroblasts present.
4. There was no significant difference in invasiveness in the presence of senescent relative to presenescent fibroblasts for two breast epithelial cell lines tested.