The Gene Expression Program of Prostate Fibroblast Senescence Modulates Neoplastic Epithelial Cell Proliferation through Paracrine Mechanisms

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Abstract
The greatest risk factor for developing carcinoma of the prostate is advanced age. Potential molecular and physiologic contributors to the frequency of cancer occurrence in older individuals include the accumulation of somatic mutations through defects in genome maintenance, epigenetic gene silencing, oxidative stress, loss of immune surveillance, telomere dysfunction, chronic inflammation, and alterations in tissue microenvironment. In this context, the process of prostate carcinogenesis can be influenced through interactions between intrinsic cellular alterations and the extrinsic microenvironment and macroenvironment, both of which change substantially as a consequence of aging. In this study, we sought to characterize the molecular alterations that occur during the process of prostate fibroblast senescence to identify factors in the aged tissue microenvironment capable of promoting the proliferation and potentially the neoplastic progression of prostate epithelium. We evaluated three mechanisms leading to cell senescence: oxidative stress, DNA damage, and replicative exhaustion. We identified a consistent program of gene expression that includes a subset of paracrine factors capable of influencing adjacent prostate epithelial growth. Both direct coculture and conditioned medium from senescent prostate fibroblasts stimulated epithelial cell proliferation, 3-fold and 2-fold, respectively. The paracrine-acting proteins fibroblast growth factor 7, hepatocyte growth factor, and amphiregulin (AREG) were elevated in the extracellular environment of senescent prostate fibroblasts. Exogenous AREG alone stimulated prostate epithelial cell growth, and neutralizing antibodies and small interfering RNA targeting AREG attenuated, but did not completely abrogate the growth-promoting effects of senescent fibroblast conditioned medium. These results support the concept that aging-related changes in the prostate microenvironment may contribute to the progression of prostate neoplasia. (Cancer Res 2006; 66(2): 794-802)

Introduction
Clinical prostate cancer is extremely rare in men ages <40, occurring with a frequency of 1 in 10,000 individuals (1). Unfortunately, the incidence increases dramatically over the ensuing decades to represent the most common noncutaneous malignancy in men >60 years of age, with a one-in-seven chance of cancer detection between ages 60 and 79 years. This relationship between prostate cancer incidence and aging is consistent across ethnic and racial groups. The prevalence of latent or indolent prostate carcinoma also increases in a dramatic fashion with aging. Sakr et al. (2) systematically examined prostate glands from young males and identified prostatic intraepithelial neoplasia in 0%, 9%, 20%, and 44%, and foci of histologic cancer in 0%, 0%, 27%, and 34% in the second, third, fourth, and fifth decades of age, respectively. Understanding the factors influencing the progression of these cancers to invasive and lethal forms represents an active area of research. Although secondary and tertiary events in the initiated epithelium contribute cellular characteristics driving neoplastic progression, it is also apparent that reactive or aging-related alterations in the tumor microenvironment provide necessary or sufficient influences that promote tumor cell invasion and metastasis.

The host microenvironment is increasingly viewed as an important active contributor to tumor growth and tumor suppression. Sternlicht et al. (3) reported that manipulation of the microenvironment through overexpression of stromelysin 1 could produce carcinomas derived from the adjacent parenchymal cells. Malignant breast epithelial cells can be epigenetically reprogrammed to a near-normal morphology with the appropriate microenvironment in vitro (4, 5). Conversely, morphologically normal breast tissue can exhibit invasive growth in vivo through alterations in the stromal environment (6). Exposure of mammary gland stroma to irradiation or carcinogens has been shown to promote tumor formation by nontumorigenic breast epithelial cells (7, 8), whereas normal breast stroma does not support tumorigenesis. Thus, the microenvironment provided by the stroma can be a powerful suppressor—or promoter—of malignant epithelial phenotypes caused by oncogenic mutations. In the context of prostate cancer, elegant studies by Olumi et al. (9) have shown that nontumorigenic prostate epithelial cells can become tumorigenic when cocultured with fibroblasts obtained from regions near tumors. Inactivation of the transforming growth factor-β (TGF-β) type II receptor gene in mouse fibroblasts resulted in intraepithelial neoplasia in the prostate and invasive cancers of the forestomach (10), a finding that further supports the important role of stroma in the process of carcinogenesis.

There is substantial evidence that aging-related changes can influence stromal-epithelial interactions leading to an environment permissive for neoplastic growth. Cellular senescence represents an aging-associated process and senescent cells accumulate in tissues with age (11, 12). Although senescent and tumor-associated
reactive fibroblasts differ in growth potential and morphology, they share the ability to stimulate the proliferation and invasive behavior of initiated epithelial cells through direct contact or secreted factors. Work by Krtolica et al. (13) has shown the ability of senescent human prostate fibroblasts to promote the growth and tumorigenesis of premalignant and malignant breast epithelial cells, a finding that provides a mechanistic link between stromal aging and carcinogenesis.

In this study, we sought to characterize the molecular alterations that occur during the process of prostate fibroblast senescence to identify factors in the aged tissue microenvironment capable of promoting the proliferation and potentially the neoplastic progression of prostate epithelium. We evaluated three mechanisms leading to cell senescence (i.e., oxidative stress, DNA damage, and replicative exhaustion) and identified a common and consistent program of gene expression that includes a subset of paracrine factors capable of influencing adjacent prostate epithelial growth. These results support the concept that aging-related changes in the prostate microenvironment may contribute to the genesis and progression of prostate neoplasia.

Materials and Methods

Cell culture. The methods for isolating and propagating the primary human prostate stromal cells used in this study were described previously (isolates PSC27, PSC31, and PSC36; ref. 14). Briefly, tissues from benign areas of radical prostatectomy specimens were collected under approval by the institutional review board. Stromal cells were cultured in PSC medium [80% MCDR131 (Invitrogen, Carlsbad, CA) supplemented with 10% FCS, nonessential amino acids, insulin, dexamethasone, transferrin, selenium, and 20% AmnioMax (Life Technologies, Carlsbad, CA)], and routinely subcultured 1:8. The human prostatic epithelial cell line BPH1 was derived from nonmalignant prostatic tissue with benign hyperplasia and immortalized by transfection with SV40-large T antigen and has been routinely subcultured 1:8. The neoplastic metastatic M12 human prostate epithelial cell line and culture conditions have been described previously (16). BPH1 and M12 cells were transfected with pRES2-EFGP (BD Bioscience, Palo Alto, CA) using LipofectAMINE 2000 (Invitrogen) according to the recommendations of the manufacturer. The cells were passaged 1:10 the next day into fresh medium and subsequently flow sorted in a FACSVantage (Becton Dickinson, Palo Alto, CA) with selection for green fluorescent protein (GFP) expression. Positive cells were seeded into DMEM + 10% FCS, expanded, and stored frozen in liquid nitrogen. These sublines were designated BPH1-GFP2 and M12-GFP2 and routinely subcultured under the same culture conditions as the parental cells. Human prostate epithelial cell lines DU145 and PC3 were routinely subcultured under the same culture conditions as BPH1 and used without modifications.

Senescence induction. Normal human prostatic stromal cells (PSC27, PSC31, and PSC36) were grown in PSC medium until 80% confluent. The cells were then treated for 2 hours at 37°C with 1 mmol/L hydrogen peroxide (H2O2) as described by Chen et al. (17) or overnight with 100 μg/ml bleomycin in PSC medium. After treatment, the cells were rinsed twice with PBS and left to recover 3 days in PSC medium. Following recovery, cells were designated PSC27ASB (PSC27 accelerated senescence by bleomycin), PSC27ASH (PSC27 accelerated senescence by H2O2), or PSC27N (presenescence PSC27). Hydrogen peroxide and bleomycin were purchased from Sigma-Aldrich Biotechnology LP (St. Louis, MO).

To generate cells at replicative senescence, PSC27 cells were cultured until cell doubling time >2 weeks (~45 cell doublings). At this time, the cells were considered to have reached replicative exhaustion and designated PSC27RS. Induction of senescence was verified by measuring the increase in senescence-associated β-galactosidase (β-Gal) activity essentially as described previously (11). Briefly, cells were washed in PBS and fixed 3 minutes in 10% neutral buffered formalin (Sigma, St. Louis, MO). The fixed cells were then washed in PBS and stained overnight in 1 mg/ml 5-bromo-4-chloro-3-indolyl β-D-galactoside, 40 mmol/L citric acid/sodium phosphate (pH 6.0), 5 mmol/L potassium ferricyanide, 5 mmol/L potassium ferrocyanide, 150 mmol/L NaCl and 2 mmol/L MgCl2. Expression of the β-Gal transcript was also quantified by PCR (see below).

In vitro cocultures of epithelium and fibroblasts. BPH1-GFP2 cells were mixed with various proportions of PSC27N, PSC27ASB, PSC27ASH, or PSC27RS using cell numbers previously determined to form confluent lawns of fibroblasts in six-well plates. The fibroblast ratios were as follows: 100% PSC27N, 10% PSC27ASB/90% PSC27N, 30% PSC27ASB/70% PSC27N, 100% PSC27ASB, 10% PSC27ASH/90% PSC27N, 30% PSC27ASH/70% PSC27N, 100% PSC27ASH, 10% PSC27RS/90% PSC27N, 30% PSC27RS/70% PSC27N, and 100% PSC27RS. Each cell mixture was seeded in a six-well plate (20,000 BPH1-GFP2 cells per well) in DMEM with 0.5% FBS. The cultures were incubated for 3 days after which cells were detached with trypsin and the total cell number was determined by direct counting in a hemacytometer. The PSC/BPH1-GFP2 proportion was determined on a FACScan (Becton Dickinson) using GFP fluorescence as a marker for BPH1-GFP2 cells. M12-GFP cells were mixed with PSC27N or PSC27ASH to form confluent lawns of fibroblasts in six-well plates and analyzed as above. The means of the cell quantitation results from each experimental condition were compared using a two-sample Student’s t test assuming unequal variances.

Culture of neoplastic prostate epithelial cells with senescent fibroblast-conditioned medium and amphiregulin. Confluent cultures of PSC27N, PSC27ASB, PSC27ASH, and PSC27RS were rinsed thrice with PBS and incubated for 3 days in DMEM + 0.5% FCS. The supernatant was harvested and stored frozen at −80°C. Conditioned medium was thawed and diluted 1:1 with fresh DMEM + 0.5% FCS before use. BPH1-GFP2, DU145, or PC3 cells were seeded at 20,000 per well in six-well plates in conditioned medium or fresh DMEM + 0.5% FCS. The cultures were incubated for 3 days and the total number of cells was determined by direct counting in a hemacytometer.

To evaluate the effect of amphiregulin (AREG) on prostate epithelial cell growth, 20,000 BPH1-GFP2 cells were seeded per well in six-well plates in DMEM + 0.5% FCS containing increasing concentrations of AREG (0, 10−9, and 10−8 mol/L). The cultures were incubated for 3 days and the cell numbers were quantitated. Separately, 20,000 BPH1-GFP2 cells were seeded per well in six-well plates in PSC27ASH and PSC27N-conditioned medium containing 100 ng/ml neutralizing anti-AREG antibodies (mouse monoclonal anti-human AREG IgG, MAB362; R&D Systems, Inc., Minneapolis, MN) or control mouse IgG. The cultures were incubated for 3 days, and the cell numbers were quantitated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay.

Quantitative reverse transcription-PCR. Total cellular RNA was isolated from cultured cells using the TRizol reagent (Invitrogen) and 2 μg of total RNA was reverse transcribed using SuperScriptII Reverse Transcriptase (Invitrogen) according to the recommendations of the manufacturer. The DNA was then hydrolyzed 15 minutes at 65°C in 0.20 mol/L NaOH and 0.10 mol/L EDTA before neutralization with 0.33 mol/L Tris (pH 7.4). The cDNA was purified with a Qiagen (Valencia, CA) PCR clean-up column according to the recommendations of the manufacturer. Primers specific for the genes of interest were designed using the web-based primer design service Primer3 provided by the Whitehead Institute for Biomedical Research (http://fokker.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). Before quantitative PCR analysis, the suitability of the PCR primers were examined using normal human prostate cDNA, Biolase Taq polymerase (Bioline, Foster City, CA), and the GeneAmp PCR system 9700 (Applied Biosystems, Randolph, MA). Briefly, 1 ng template cDNA was amplified with 0.3 μmol/L primers in 30 cycles of 94°C (15 seconds), 60°C (30 seconds), and 72°C (30 seconds). The PCR products were analyzed on a 4% agarose gel in 1× TAE with 5 μL 10 mg/ml ethidium bromide per 100 μl gel. The following primer pairs generated strong unique PCR products of the appropriate lengths and were selected for use in quantitative PCR reactions. Human glyceraldehyde-3-phosphate dehydrogenase (GAPDH; control):
ACTTCAACGGACACCACACT (forward primer) and CACCCGTGTTGCTG-TAGCCA/AA (reverse primer). Human β-actin (control): AAGGAGAATG GCCAGTCCT (forward primer) and TGCTATCACCTCCCTGGTGTG (reverse primer). Human α-tubulin (control): GGTTAGGTGGTTCCTAAAG (forward primer) and GGTTGGTATGCTGAAATGG (reverse primer). Human manganese superoxide dismutase 2 (MnSOD2): AGTGAACGAAACAGAGCGCC (forward primer) and TCCACACACATACCCCC (reverse primer). Human AREG: TGGA- TTGGACCTCTAATGACA (forward primer) and AGGCCAGTTATTTGCTG-TCG (reverse primer). Human fibroblast growth factor 7 (FGF7): CTTGAGATCGATAAAAGAGGCAC (forward primer) and ATTCTTCTACCTCTTG-GTCTCCTT (reverse primer). Human hepatocyte growth factor (HGF): GTCTCCTGTTGCTGTGATGTC (forward primer) and TCGGACAAAATAC- CAGGAC (reverse primer). Relative quantification of gene expression by quantitative PCR (40 cycles of 60°C annealing, 72°C extension and 95°C melting) was done on a 7700 Sequence Detector (ABI, Foster City, CA) using SYBR Green Master mix (ABI) and gene-specific primers according to the recommendations of the manufacturer.

cDNA microarray analysis. Custom Prostate Expression Database
cDNA microarrays were constructed as previously described (18) using clones derived from the Prostate Expression Database, a sequence repository of human prostate expressed sequence tag data available to the public (www.pedb.org; ref. 19). A second microarray was constructed using ∼17K cDNAs chosen from the Research Genetics sequence-verified set of IMAGE clones. The inserts of individual cDNA clones were amplified by PCR, purified, and spotted in duplicate onto glass microscope slides (Gold Seal, Becton Dickinson) with a robotic spotting tool (GeneMachine OmniGrid 100). Probes were generated from the PSC27, PSC31, and PSC36 prostate fibroblast cell lines at steady state and following induction of senescence. Labeling with Cy3 and Cy5 fluorescent dyes and hybridization to the microarray slides were essentially as described (20).

Fluorescent array images were collected for both Cy3 and Cy5 using a GenePix 4000 B fluorescent scanner (Axon Instruments, Foster City, CA). The image intensity data were gridded and extracted using GENEPIX PRO 4.1 software (Axon Instruments), and spots of poor quality determined by visual inspection were removed from further analysis. All three stromal cell lines (PSC27, PSC31, and PSC36) and three senescence-inducing treatments [H2O2 treatment (ASH), bleomycin treatment (ASB), and replicative visual inspection were removed from further analysis. All three stromal cell lines were essentially as described (20).

Normalization of the Cy3 and Cy5 fluorescent signal on each array was done using the significance analysis of microarray procedure (http://www-stat. stanford.edu/~tibs/SAM/; ref. 21). A one-sample t test was used to determine whether the mean gene expression of all cell lines and all treatments differed from zero. Gene expression differences with a false discovery rate of ≤5% were considered significant. A multiclass test (one-way ANOVA) in Significance Analysis of Microarray was used to assess the differences between cell isolates.

Western blot analysis. Confluent cultures of PSC27N, PSC27ASH, PSC31N, and PSC31ASH in T150 flasks were rinsed thrice with PBS and the cultures were incubated for 3 days in DMEM with 0.5% FBS. The supernatant was harvested and stored frozen at −80°C. The protein was concentrated before SDS-PAGE by trichloroacetic acid precipitation as follows: 1 ml conditioned medium was precipitated for 1 hour on ice with 10% final trichloroacetic acid concentration. The tube was spun in microcentrifuge at 14,000 rpm for 45 minutes at 4°C. The supernatant was removed and the pellet was washed with 1 mL cold acetone. The centrifugation and acetone wash was repeated. After drying, the sample was dissolved in loading buffer, reduced by boiling with 1% DTT for 5 minutes, and separated on a NuPAGE MES 4% to 12% gel (Invitrogen) according to the instructions of the manufacturer. Purified recombinant human AREG (R&D Systems) was included as a positive control at quantities of 0.1 and 0.02 μg. The separated proteins were transferred to nitrocellulose membranes (Invitrogen) in a trans-blot semidry transfer cell (Bio-Rad, Hercules, CA). Nonspecific protein binding to the nitrocellulose membranes was saturated overnight with 3% dry milk, 2% bovine serum albumin, and 0.1% Tween 20 in PBS (Blotto). The filters were then blotted with antibodies to AREG, FGF7 (R&D Systems), or HGF (Sigma) in Blotto for 3 hours at room temperature. Adhered primary antibodies were visualized with horseradish peroxidase–conjugated ImmunoPure secondary antibodies (Pierce, Rockford, IL) and the SuperSignal West Pico Staining system (Pierce) according to the instructions of the manufacturer. Western blot analysis was repeated with a second antibody recognizing AREG (AF262, R&D Systems).

Results

Induction of prostate fibroblast senescence. Several factors have been shown to induce a phenotype of cellular senescence (23, 24). In this study, we evaluated three senescence mechanisms that prostate fibroblasts could reasonably encounter in their natural environment: oxidative stress (17), DNA damage due to chemotherapeutic exposure (25), and replicative exhaustion (26). We studied three independent primary prostate stromal cell isolates (PSC27, PSC31, and PSC32) to determine both the consistency and variability of the phenotypic and gene expression features of the senescence program in this cell type. To verify a senescence phenotype associating with
each mechanism, we visually inspected cell cultures for morphologic features of senescence and measured expression of β-Gal by quantitative reverse transcription-PCR (qRT-PCR) and by staining at pH 6 (SA-β-Gal; ref. 11).

Primary prostate fibroblast isolates were treated with 1 mmol/L H$_2$O$_2$, 100 μg/mL bleomycin, or grown to replicative senescence. Morphologic changes previously associated with senescence, including cell enlargement and flattening, were clearly apparent (11, 13). SA-β-Gal staining was not observed in presenescent cell cultures but was readily visualized following each of the three treatments (data not shown). To provide a more quantitative measure, β-Gal and SOD2 transcripts were analyzed by qRT-PCR before and after treatments and compared relative to GAPDH gene expression. β-Gal expression increased 4.3-fold after ASH, 2.8-fold after ASB, and 2.3-fold in RS, relative to low-passage presenescent cells (Fig. 1). To ensure that these changes were due to senescence and not growth quiescence, PSC27 and PSC31 fibroblast isolates were cultured to confluence and further incubated 7 days under normal culture conditions. The quiescent PSC27 and PSC31 did not exhibit increases in β-Gal mRNA or SA-β-Gal staining compared with proliferating cells (data not shown).

The transcriptional program of prostate fibroblast senescence. To characterize common and unique features of the senescence program in prostate fibroblasts, we used cDNA microarray analysis to quantitate transcript abundance levels...
between presenescent PSC27, PSC31, and PSC36 fibroblasts and parallel cultures induced to senesce by H2O2, bleomycin, or replicative exhaustion. A one-sample t test comparing transcript abundance levels across a matrix of three fibroblast isolates and three senescence mechanisms—a total of nine experiments—identified 1,073 clones (representing 855 unique genes, 714 of which are characterized) with significant differential expression results across the nine senescent samples compared with presenescent controls (false discovery rate ≤ 1%). The consistency of these results is supported by direct comparisons of the three different senescence mechanisms to each other in which no clones were significantly differentially regulated (< 1% false discovery rate). A comparison of the three different fibroblast isolates to each other at steady state determined that 436 clones were differentially expressed (< 1% false discovery rate, one-way ANOVA). Although the expression of these genes differed significantly between cell isolates, upon further analysis, it was determined that although measurable, the differences were due to relatively small magnitudes of expression between the fibroblast isolates; on the other hand, the direction of expression changes for the vast majority of genes was concordant between all cell lines and treatments.

To prioritize genes for further study, we first sought to identify those with consistent alterations across cell lines and treatments, and exhibited magnitudes of increased transcriptional changes that might reasonably be measured at the protein level. Of the cDNAs represented on the Prostate Expression Database microarrays, 122 genes with significant expression changes also exhibited average fold changes of ≥ 2 in senescent versus presenescent cells (Fig. 2A). Additional profiling experiments using a larger clone set identified 588 additional genes with statistically significant gene expression alterations of ≥ 2-fold across the three fibroblast isolates following H2O2 treatment. We chose to focus on the 407 genes with elevated expression. Of these, we were particularly interested in the subset of 71 genes that encode extracellular proteins annotated in the Genome Ontology database with the potential to influence adjacent cells via paracrine mechanisms (Fig. 2B). The identification of senescence-associated alterations in genes with documented roles as paracrine mediators of cell proliferation prompted further studies designed to confirm the microarray results for several genes in this functional category. We used quantitative RT-PCR to measure the senescence-associated increase of transcripts encoding AREG, cytokine-like protein C17 (C17), chemokine C-X-C motif-ligand 1 (CXCL1), FGF7, HGF, insulin-like growth factor binding protein (IGFBP) 2, IGFBP3, IGFBP5, interleukin 8 (IL-8), laminin β1 (LAMB1), matrix metalloproteinase 2 (MMP2), and tissue inhibitor of matrix metalloproteinase 1 (TIMP1). The mRNA abundance in senescent (PSC27ASH) and presenescent (PSC27N) cells from two unrelated experiments were normalized against GAPDH and were compared (Table 1). For each gene, the senescence-associated increase in expression originally measured by microarray hybridization was confirmed by qRT-PCR in a replicate biological experiment. The IGFBP mRNAs increased between 26- and 53-fold following senescence. IL-8, MMP2, and TIMP2 increased 22-, 16-, and 14-fold, respectively.

### Table 1. Verification of transcript alterations in senescent versus presenescent prostate fibroblasts

<table>
<thead>
<tr>
<th>Gene symbol</th>
<th>Gene description</th>
<th>Fold-change: microarray</th>
<th>Fold-change: qRT-PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>AREG</td>
<td>Amphiregulin</td>
<td>4.4</td>
<td>3.4</td>
</tr>
<tr>
<td>C17</td>
<td>Cytokine-like protein C17</td>
<td>5.3</td>
<td>2.2</td>
</tr>
<tr>
<td>CXCL1</td>
<td>Chemokine (C-X-C motif) ligand 1</td>
<td>3.4</td>
<td>8.2</td>
</tr>
<tr>
<td>FGF7</td>
<td>Fibroblast growth factor 7</td>
<td>2.8</td>
<td>2.5</td>
</tr>
<tr>
<td>HGF</td>
<td>Hepatocyte growth factor</td>
<td>2.5</td>
<td>5.1</td>
</tr>
<tr>
<td>IGFBP2</td>
<td>Insulin-like growth factor binding protein 2</td>
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</tr>
<tr>
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<td>Interleukin 8</td>
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</tr>
<tr>
<td>LAMB1</td>
<td>Laminin β1</td>
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</tr>
<tr>
<td>MMP2</td>
<td>Matrix metalloproteinase 2</td>
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</tr>
<tr>
<td>TIMP1</td>
<td>Tissue inhibitor of metalloproteinase 1</td>
<td>2.6</td>
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</table>

The identification of senescence-associated alterations in genes binding protein (IGFBP) 2, IGFBP3, IGFBP5, interleukin 8 (IL-8), laminin β1 (LAMB1), matrix metalloproteinase 2 (MMP2), and tissue inhibitor of matrix metalloproteinase 1 (TIMP1). The mRNA abundance in senescent (PSC27ASH) and presenescent (PSC27N) cells from two unrelated experiments were normalized against GAPDH and were compared (Table 1). For each gene, the senescence-associated increase in expression originally measured by microarray hybridization was confirmed by qRT-PCR in a replicate biological experiment. The IGFBP mRNAs increased between 26- and 53-fold following senescence. IL-8, MMP2, and TIMP2 increased 22-, 16-, and 14-fold, respectively.

**Evaluation of paracrine mediators of neoplastic epithelial cell growth.** The identification of senescence-induced expression of transcripts encoding fibroblast proteins with the potential to influence epithelial proliferation prompted experiments designed to determine if senescent prostate fibroblasts could stimulate the growth of immortalized prostate epithelial cells when in close proximity. Primary prostate fibroblasts induced to senesce with H2O2, bleomycin, or replicative exhaustion were each cocultured with BPH1-GFP2 cells for 72 hours. To ensure that the coculture conditions of BPH1 with nonproliferating senescent fibroblasts were similar to that of untreated proliferation-competent fibroblasts, enough fibroblasts were used to form a confluent layer immediately upon seeding without proliferation. PSC27ASH or PSC27ASB senescent fibroblasts stimulated the proliferation of the BPH1-GFP2 epithelial cells ~ 2.9-fold each relative to coculture with untreated PSC27N cells (P < 0.001 and P < 0.001, respectively; Fig. 3A and C). Senescent fibroblasts comprising 30% of the entire fibroblast population was sufficient to exert measurable effects on epithelial cell proliferation (Fig. 3C). PSC27RS senescent fibroblasts also stimulated epithelial cell proliferation although to a lesser extent (1.5-fold; P < 0.001; Fig. 3B). The stimulating effect of senescent fibroblasts was corroborated by the finding that PSC27ASH also stimulated the proliferation of the highly metastatic M12-GFP epithelial cells ~ 1.3-fold relative to coculture with untreated PSC27N cells (P = 0.02; Fig. 3D).

We next sought to determine if the influence of senescent prostate fibroblasts on epithelial growth resulted from soluble factors. We generated conditioned medium from presenescent PSC27N cells and senescent PSC27ASH, PSC27RS cells, and measured BPH1-GFP2 cell numbers after growth for 3 days in the different conditioned media. The proliferation of BPH1-GFP2 cells was stimulated 1.8-fold (P = 0.004) and 1.6-fold (P = 0.007) with medium from PSC27ASH
and PSC27RS, respectively, when compared with medium conditioned with PSC27N cells (Fig. 4A). Consistent with these findings, conditioned medium from the senescent PSC27ASH stimulated the growth of tumorigenic DU145 and PC3 cells 1.2-fold ($P < 0.001$) relative to conditioned medium from presenescent PSC27N fibroblasts (Fig. 4B). These results suggest that a significant component of the senescent fibroblast proliferative influence toward epithelium is mediated through soluble factors.

**Identification of AREG as a senescence-associated factor modulating the proliferation of neoplastic prostate epithelium.**

To confirm that senescence-associated transcript alterations produced corresponding changes in extracellular protein levels, we evaluated conditioned medium obtained from senescent PSC27 fibroblasts for the presence of FGF7, HGF, and AREG by Western blot analysis (Fig. 5A). The FGF7 antibody detected a strongly reactive protein with a molecular mass of ~25 kDa and a weakly reactive protein of molecular mass 75 kDa. Higher amounts of the 25 kDa protein were measurable in medium conditioned by senescent prostate fibroblasts compared with presenescent cells, whereas the 75 kDa protein was not appreciably altered. A 28 kDa form of FGF7 has previously been detected in medium conditioned by human embryonic lung fibroblasts (27). Western analysis of conditioned culture medium with an antibody recognizing HGF detected several proteins with apparent molecular masses of 53, 35, and 30 kDa, and a faintly visualized protein with an apparent molecular mass of ~75 kDa. The weakly staining high molecular mass band is consistent with the intact precursor pro-HGF protein, whereas the lower molecular mass bands correspond to the heavy and light chains (28). Significantly higher amounts of all four proteins were detected in medium conditioned by senescent prostate fibroblasts cells compared with presenescent cells.

The AREG antibody detected a protein in fibroblast-conditioned medium with an apparent molecular mass of ~58 kDa. Considerably greater amounts of the protein were detected in medium conditioned by senescent fibroblasts compared with presenescent cells (Fig. 5A). Although the predicted molecular mass of AREG is ~20 kDa, size ranges of 60, 55, 28, 18, and 10 kDa have been found in medium conditioned by the human breast cancer cell line MCF-7 due to glycosylation (29). AREG sizes of 51 and 27 kDa have been measured in microsomal preparations from sheep mammary glands (30). To verify antibody specificity, we ran 0.1 µg of recombinant AREG and measured a predominant band at ~60 kDa and a faint band at ~20 kDa (Fig. 5A). A Western blot run with 0.02 µg AREG resulted in a shift of the predominant band to ~20 kDa. Repeating the Western analysis with a second AREG-specific antibody produced similar results (data not shown).

AREG has been shown to be expressed in prostate interstitial smooth muscle cells and to stimulate the proliferation of primary benign prostate epithelium (31). AREG expression has also been

![Figure 3](attachment:image.jpg)
shown to be up-regulated in prostate cancers with altered subcellular localization patterns (32). To determine if AREG contributed to a component of the proliferative effect of senescent prostate fibroblasts toward prostate epithelium, we added recombinant AREG to BPH1-GFP2 cells for 72 hours and quantitated cell numbers. AREG concentrations of $1 \times 10^{-8}$ mol/L stimulated the proliferation of BPH1-GFP2 cells 2.4-fold relative to cells grown in the absence of AREG ($P < 0.01$; Fig. 5D). Senescent fibroblast conditioned medium treated with anti-AREG-neutralizing antibodies lost a small but significant component of the growth-promoting effects relative to complete conditioned medium ($P = 0.002$; Fig. 5C). To corroborate these findings, we treated fibroblasts with AREG or control RNA interference (RNAi).

The senescence-induced expression of AREG was not noticeably affected by control RNAi but was reduced 46-fold by AREG RNAi (Fig. 5D). Conditioned medium from senescent fibroblasts treated with AREG-specific siRNA lost a small but significant component of the growth-promoting effects ($P = 0.04$; Fig. 5E). These results suggest that multiple paracrine-acting factors secreted or liberated from senescent prostate fibroblasts contribute to epithelial growth stimulation.

Discussion

The “host” microenvironment is increasingly viewed as an important active contributor to tumor growth and tumor suppression. The somatic mutation theory of cancer postulates that carcinomas result from a single somatic cell that accumulates multiple DNA mutations or chromosomal alterations over time: genotypic changes result in phenotypes of uncontrolled growth (33). This concept explains many aspects of tumorigenesis but other important observations indicate that additional influences must be operative. For example, embryos transplanted into ectopic sites (e.g., peritoneal cavity) can behave like malignant neoplasms (e.g., teratocarcinomas). Conversely, embryonal carcinoma cells injected into murine blastocysts contribute to normal tissues and organs in cancer-free adult mice (34). Thus, the microenvironment provided by the stroma can be a powerful suppressor—or promoter—of malignant phenotypes caused by oncogenic mutations. This is true both during embryogenesis and in adult tissues (reviewed in ref. 35).

The importance of cellular context as a contributor to carcinogenesis has led to alternative explanations for the development and progression of epithelial malignancies. One hypothesis, designated the Tissue Organization and Field Theory (reviewed in ref. 36), builds on an extensive body of work in embryology involving morphogenetic fields of cellular interactions that collectively dictate cellular differentiation and tissue organization through cellular contacts and diffusible gradients of morphogens (37–39). In agreement with the Tissue Organization and Field Theory are observations that tumors exhibit striking similarities to “wounds that do not heal” (40). The “reactive” stroma surrounding carcinomas display morphologic and biochemical characteristics akin to changes associated with wound healing: fibroblast and epithelial proliferation, cell migration, recruitment of inflammatory cells, and angiogenesis. Recent studies using microarray-based expression profiling have shown striking similarities between signatures of fibroblast serum response and human tumors (41). Whereas the concept of a wound-associated or reactive stroma implies that the microenvironment is altered in response to an extrinsic (e.g., epithelial) event, it is also plausible that intrinsic stromal alterations are operative as primary or permissive events allowing for, or magnifying, a reactive phenotype. Studies of the tumor-promoting effects of senescent fibroblasts on breast carcinogenesis and the findings reported here suggest that age-dependent stromal processes operate as a tissue-modifying field effect.

The substantial number of reproducible molecular changes identified in this study that associate with prostate fibroblast senescence includes a host of extracellular proteins with well-described capabilities for influencing the growth or survival of cells in the locoregional environment. Paracrine-acting proteins identified in our study included HGF/scatter factor, a protein originally identified as a fibroblast-derived epithelial cell motility factor and a potent mitogen of hepatocytes (42). HGF has been shown to disrupt epithelial cell morphogenesis, regulate the breakdown of cell-to-cell junctions, and stimulate the migration and invasion of single cells (43, 44). HGF expression has been shown to increase in skin fibroblasts from old individuals and in response to IGFs that increase with aging (45, 46). HGF can coactivate androgen receptor signaling, leading to androgen-independent prostate cancer growth (47, 48). The important influence of stroma and HGF on the development of neoplastic growth was recently shown in experiments analyzing mice with...
targeted deletions of the TGF-β type II receptor specifically in fibroblasts. These mice developed invasive gastric cancers and prostate intraepithelial neoplasia through a mechanism involving stromally derived HGF (10).

Other senescence-induced mitogenic factors identified in this study include FGF7/KGF, IGFBPs, and AREG. AREG is a heparin-binding member of the epidermal growth factor family that influences the survival, growth, or progression of multiple human cancers, including myeloma (49), breast (50), lung (51), pancreas (52), and prostate (31, 32). AREG has been shown to function as a survival factor, protecting hepatocytes from Fas-mediated liver injury, and cooperating with IGF-I to inhibit apoptosis of lung carcinoma cells through phosphorylation of Bad (51). In the context of prostate carcinogenesis, AREG mediates androgen-stimulated cell proliferation in the LNCaP prostate cancer cell line, suggesting the presence of an androgen-regulated autocrine loop involving the epidermal growth factor receptor and AREG. The important role for AREG in mediating the survival of prostate cancer cells to castration was recently shown through studies reporting a 5-fold increase in tumor AREG protein expression following androgen depletion (53). Blockade of the HER1 tyrosine kinase receptor in conjunction with castration significantly increased tumor involution compared with castration alone.

In summary, the molecular signature of prostate fibroblast senescence includes a cohort of factors capable of influencing the survival and proliferation of adjacent prostate epithelium. The local production of these mitogens and prosurvival factors could exert important affects on preneoplastic lesions originally instigated by predisposing genetic variables, carcinogens, or chronic inflammation (54). Further, these findings suggest an explanation for the paradoxical age-associated increases in hormonally driven prostatic diseases, such as cancer and benign hyperplasia in the setting of age-associated declines in testosterone, whereby increases in mitogens from the aged stroma substitute for androgen loss.

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