

# Secretion of Vascular Endothelial Growth Factor by Primary Human Fibroblasts at Senescence\*

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Cellular senescence prevents the proliferation of cells at risk for neoplastic transformation. Nonetheless, the senescence response is thought to be antagonistically pleiotropic and thus contribute to aging phenotypes, including, ironically, late life cancers. The cancer-promoting activity of senescent cells is likely due to secreted molecules, the identity of which remains largely unknown. Here, we have shown that senescent fibroblasts, much more than presenescent fibroblasts, stimulate tumor vascularization in mice. Weakly malignant epithelial cells co-injected with senescent fibroblasts had larger and greater numbers of blood vessels compared with controls. Accordingly, increased vascular endothelial growth factor (VEGF) expression was a frequent characteristic of senescent human and mouse fibroblasts in culture. Importantly, conditioned medium from senescent fibroblasts, more than medium from presenescent cells, stimulates cultured human umbilical vein endothelial cells to invade a basement membrane, a hallmark of angiogenesis. Increased VEGF expression was specific to the senescent phenotype and increased whether senescence was induced by replicative exhaustion, overexpression of p16<sup>Ink4a</sup>, or overexpression of oncogenic RAS. The senescence-dependent increase in VEGF production was accompanied by very little increase in hypoxic-inducible (transcription) factor 1  $\alpha$  protein levels, and hypoxia further induced VEGF in senescent cells. This result suggests the rise in VEGF expression at senescence is not a hypoxic response. Our findings may in part explain why senescent cells stimulate tumorigenesis *in vivo* and support the idea that senescent cells may facilitate age-associated cancer development by secreting factors that promote malignant progression.

In complex organisms such as mammals, diverse stress can induce proliferative cells to either die by programmed cell death (apoptosis) or irreversibly withdraw from the cell cycle by a

process termed cellular senescence. Both responses most likely evolved to suppress the proliferation of dysfunctional or damaged cells and hence the possibility of oncogenic transformation (1). Whether a cell undergoes apoptosis or senescence can have important, yet different, physiological consequences, especially over the life span. Apoptosis effectively eliminates defective cells but can eventually lead to tissue atrophy. By contrast, senescent cells generally resist apoptosis and thus can accumulate *in vivo* (2–5); however, because they secrete biologically active molecules, these irreversibly growth-arrested cells can alter the behavior of neighboring cells (reviewed in Refs. 6, 7). Thus, both the apoptotic and senescence responses may be antagonistically pleiotropic, benefiting young organisms by protecting them from cancer early in life but causing detrimental effects later in life (1).

A diversity of stresses can induce cellular senescence (reviewed in Ref. 8). These include the dysfunctional telomeres that result from repeated cell division (replicative senescence), severe or irreparable DNA damage (genotoxic stress), the expression of certain oncogenes (oncogene-induced senescence), and agents or conditions that disrupt chromatin organization. Despite the disparate mechanisms by which these stresses act, senescent cells share numerous features, including an arrest of cell proliferation that cannot be reversed by physiological signals, an enlarged flattened morphology, expression of a neutral  $\beta$ -galactosidase (senescence-associated  $\beta$ -galactosidase), and an altered pattern of gene expression. The senescence response depends on the activities of two major tumor suppressor pathways, one controlled by p53 and another controlled by p16<sup>INK4a</sup> and pRB (reviewed in Ref. 6).

We have shown that senescent stromal fibroblasts secrete factors that can alter the structure and function of normal epithelial tissue structures (9). In addition, we found that senescent fibroblasts secrete factors that can stimulate the proliferation of premalignant epithelial cells in culture and facilitate their conversion to malignant tumors *in vivo* (10). This finding suggests that the senescence of stromal cells might cooperate with the acquisition of oncogenic mutations by epithelial cells to promote late life cancers (11). Indirect support for this idea derives from studies of irradiated stromal cells. Sublethal irradiation of normal breast stroma increased the incidence of breast tumors in mice (12). Likewise, irradiated fibroblasts produced factors that increased the invasiveness of pancreatic cancer cells in culture (13).<sup>2</sup> Because genotoxic stress, including

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<sup>2</sup> J. P. Coppé and J. Campisi, manuscript in preparation.

irradiation, induces a senescence response, especially in fibroblasts (14), it is likely in these studies that fibroblasts became senescent after irradiation. The identity of the cancer-promoting factors produced by senescent fibroblasts remains largely unknown.

In some respects, the phenotype of senescent fibroblasts resembles that of tumor- or carcinoma-associated fibroblasts (15), which are thought to provide factors that facilitate the ability of the developing carcinoma to invade and survive in the surrounding tissue. Recently, a mechanism by which tumor-associated fibroblasts promote cancer development was elucidated (16). This study showed that such fibroblasts might promote human breast cancer progression by secreting stromal cell-derived factor 1 (SDF-1)<sup>3</sup>. SDF-1 recruits endothelial progenitor cells and hence stimulates new blood vessel formation, or angiogenesis. Angiogenesis is considered one of six important steps required for the development of a malignant tumor (17).

Here, we have shown that senescent human lung fibroblasts may likewise promote tumorigenesis by stimulating angiogenesis but in this case by secreting elevated levels of angiogenic factors, one of which is the vascular endothelial growth factor (VEGF). We showed that fibroblast-secreted VEGF stimulates the invasiveness of human umbilical vascular endothelial cells (HUVECs) in culture and that the presence of senescent fibroblasts increased the vascularization of tumors in mice. The elevated VEGF production by senescent fibroblasts was due in part to increased VEGF mRNA levels and occurred whether cells were induced to senesce by replicative exhaustion, expression of oncogenic RAS, or overexpression of p16<sup>INK4a</sup>. Further, senescent fibroblasts had only slightly elevated levels of HIF-1 $\alpha$ , a transcription factor that induces VEGF expression in response to hypoxia (18), and retained the ability to further induce VEGF in response to hypoxia. Our findings suggest that up-regulated VEGF expression comprises part of the senescent phenotype of some human fibroblasts, independent of the hypoxic response.

## MATERIALS AND METHODS

**Cells and Cell Culture**—HUVECs (Clonetics) were cultivated in EGM-2 medium (Clonetics). Human WI-38 and IMR90 (fetal lung) and HCA2 and BJ (foreskin) fibroblasts were obtained, cultured, and passaged to senescence in atmospheric oxygen as described (19, 20). Human and mouse breast fibroblasts and EpH4-v mouse epithelial tumor cells were obtained from Dr. M. Stampfer as primary fibroblasts from patients who underwent breast reduction, and as described (9, 21). Cells were judged to be senescent as described (2, 19, 20). WI-38 cells were made quiescent by incubating for 72 h in growth medium supplemented with 0.2% fetal bovine serum as described (20). For hypoxic conditions, cells were grown in a humidified chamber adjusted to 1% oxygen.

**Vectors and Viral Infections**—Infectious virus was produced by transiently transfecting lenti and packaging vectors into 293T cells as described (22). All lentiviral constructs (lenti-GFP, lenti-p16, lenti-Ha-RAS<sup>v12</sup>) have been described (23). Non-integrating lentiviruses were generated using the same method, except that a mutated integrase (D64V) plasmid (a gift from Dr. Robert Marr, Salk Institute, La Jolla, CA) was used (24). Viral supernatants were concentrated by ultracentrifugation and titers determined by ELISA for p24, using a commercial kit (Zeptometrics). Cells were infected in the presence of 6  $\mu$ g/ml polybrene at a multiplicity of infection of 50 unless otherwise indicated. Test infections using lenti-GFP showed that a multiplicity of infection of 25 gave infection efficiencies of >99%.

**Western Blotting and ELISA Assay**—We performed Western analysis as described (19) using the following primary antibodies, actin (Chemicon MAB1501), HIF-1 $\alpha$  (BD Biosciences 610959). Secreted VEGF and SDF-1 $\alpha$  protein concentrations were measured using commercially available enzyme-linked immunosorbent assay (ELISA) kits (Quantikine; R&D Systems). Volumes of cell culture supernatants were normalized to the total number of cells present at the time of collection.

**Invasion Assay**—Conditioned medium was collected by washing cells twice with phosphate-buffered saline, followed by a 48-h incubation in serum-free Dulbecco's modified Eagle's medium. An equal number of cells were plated at the start of the experiment, and cell numbers were determined after the 48-h incubation; when necessary, conditioned medium volumes were normalized to the total number of cells present at the end of the 48-h period. 24-well format 8- $\mu$ m cell culture inserts (BD Biosciences 354578) were coated with 12.5  $\mu$ l of Matrigel (BD Biosciences 356234) diluted 1:1 with serum-free cell culture medium. The coated cell culture inserts were placed atop 300  $\mu$ l of conditioned medium or serum-free medium + recombinant human VEGF.  $1 \times 10^5$  HUVEC cells in 200  $\mu$ l of serum-free medium were added on top of the Matrigel layer. HUVECs were preincubated in serum-free medium for 12 h prior to plating on Matrigel. HUVECs were allowed to invade the Matrigel for 36 h, after which they were fixed, stained, and counted. When indicated, conditioned medium was incubated for 1 h at 4 °C in the presence of 0.5  $\mu$ g/ml anti-human VEGF antibody (R&D Systems AF-293-NA). Human recombinant VEGF (15 ng/ml) was used as a positive control.

**Tumor Studies and vWF Immunostaining**—Tumorigenic studies were performed as previously described (10). In brief, 0.75 million EpH4-v epithelial cells were injected in the presence (or absence) of one million primary mouse breast fibroblasts (MBF). 200  $\mu$ l of serum-free medium containing resuspended cells was injected subcutaneously underneath the sixth nipple. After 60 days, tumors were excised, embedded in paraffin, and stained for the von Willebrand factor (vWF) endothelial cell marker (RB-281-A; Neo Marker). In brief, tissue sections were deparaffinized, treated with proteinase K, blocked with bovine serum albumin, and incubated with the vWF primary antibody and subsequently with a biotinylated goat anti-rabbit secondary antibody. A peroxidase kit (SK-4100) from Vector Laboratories was used for detection. Tissue sections were counterstained with hematoxylin.

<sup>3</sup> The abbreviations used are: SDF-1, stromal cell-derived factor 1; VEGF, vascular endothelial growth factor; HUVEC, human umbilical vascular endothelial cell; ELISA, enzyme-linked immunosorbent assay; mBF, mouse breast fibroblast; vWF, von Willebrand factor; GFP, green fluorescent protein; HIF-1 $\alpha$ , hypoxic-inducible (transcription) factor 1  $\alpha$ .

## VEGF Secretion at Senescence

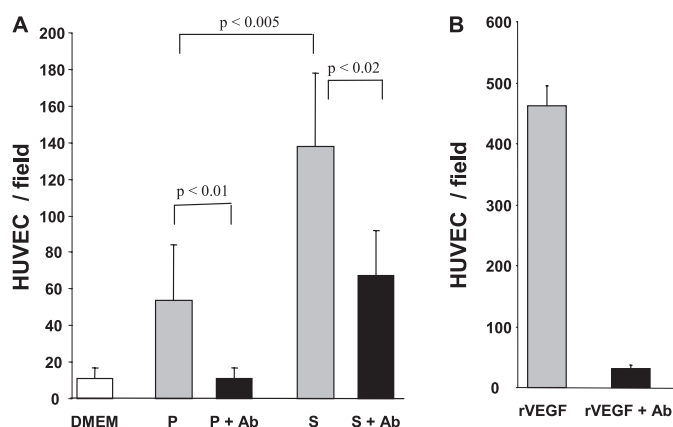
**RNA Isolation and Real-time PCR**—For RNA isolation, cells were lysed directly on the culture plate using lysis buffer provided in the RNeasy kit with DNase I digestion (Qiagen, Valencia, CA). The relative abundance of target gene mRNA, as well as the internal control 18 S rRNA, was measured by real-time quantitative polymerase chain reaction (PCR) performed on an ABI PRISM 7700 sequence detector (Applied Biosystems, Foster City, CA). Primers and probe for VEGF (all isoforms) were: upper primer, 5'-TAC-CTC-CAC-CAT-GCC-AAG-TG-3', lower primer, 5'-GAT-GAT-TCT-GCC-CTC-CTC-CTT-3', and probe, 5'-6FAM-TCC-CAG-GCT-GCA-CCC-ATG-GC-TAMRA-3'. Primers and probe for SDF-1 $\alpha$  were: upper primer, 5'-TCT-CAA-AAT-TCT-CAA-CAC-TCC-AAA-CT-3', lower primer, 5'-GCA-CAC-TTG-TCT-GTT-GTT-GTT-CTT-3' and probe, 5'-6FAM-TGC-CCT-TCA-GAT-TGT-AGC-CCG-GC-TAMRA-3'. Primers and probe for ribosomal 18 S and histone 1 $\alpha$  (H1a) genes were from the Applied Biosystems kit (4308329) and from the University of California at San Francisco core facility, respectively. Expression levels were calculated against a standard curve using serial dilutions of total RNA. Analyses were performed in quadruplicate for each sample. Values presented are the ratio of target mRNA to 18 S RNA. Statistical analyses were performed using a non-paired Student *t* test or one-way analysis of variance and considered significant when *p* was <0.05.

## RESULTS

**Conditioned Medium from Senescent Fibroblasts Stimulates HUVEC Invasiveness**—We reported that senescent WI-38 fibroblasts promote the tumorigenic progression of preneoplastic epithelial cells when co-injected into immunocompromised mice (10). The resulting tumors were large and vascularized, suggesting that angiogenesis had occurred during tumor progression. Angiogenesis is essential for the survival of tumors located >100–200  $\mu$ m from blood vessels and generally occurs because tumor and/or surrounding stromal cells secrete factors that stimulate the proliferation, migration, and invasion of surrounding endothelial cells (25). We therefore asked whether senescent WI-38 fibroblasts secrete factors that stimulate endothelial cell migration and invasion.

We collected conditioned media from presenescent and replicatively senescent fibroblasts and used them as attractants in a cell culture assay for the migration and invasion of HUVECs through basement membrane components, commercially supplied as Matrigel. Conditioned medium from senescent fibroblasts was 3-fold more potent than medium from presenescent fibroblasts in stimulating HUVEC migration and invasion (Fig. 1).

One of the most potent angiogenic factors identified to date is VEGF (26). To determine whether VEGF was responsible for the senescence-associated stimulation of HUVEC migration and invasion, we pretreated the conditioned medium with a VEGF-blocking antibody. Pretreatment of presenescent conditioned medium reduced HUVEC invasiveness to the level seen when no attractant was provided (background invasiveness) (Fig. 1A). Thus, essentially all the invasion stimulated by presenescent conditioned medium was due to VEGF. Likewise, VEGF-blocking antibody substantially reduced the ability of



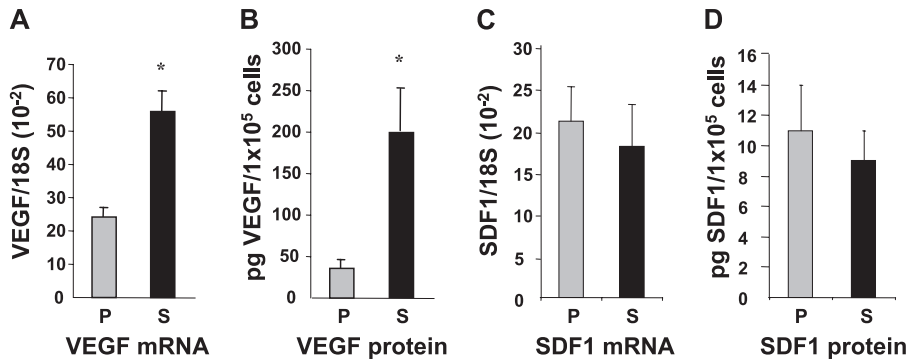
**FIGURE 1. Senescent fibroblasts produce soluble factors that stimulate invasion and migration of HUVECs.** A, human umbilical vein endothelial cells (HUVECs) were plated atop cell culture inserts coated with basement membrane components (Matrigel) and the inserts immersed in attractant medium, which consisted of serum-free medium (DMEM (Dulbecco's modified Eagle's medium), negative control) or conditioned medium collected from presenescent (P) or senescent (S) WI-38 human lung fibroblasts. Where indicated (+Ab), the medium was preincubated with VEGF-blocking antibody as described under "Materials and Methods." 36 h later, cells that migrated and invaded the Matrigel to the underside of the insert were stained and counted by light microscopy. Shown are the average number of cells/field  $\pm$  S.D. of six to eight individual cell inserts performed over two to three independent experiments. Statistical *p* values were determined using a Student *t* test. B, HUVEC migration and invasion were determined as described in panel A, except the attractant was serum-free medium containing recombinant human VEGF (rVEGF) or rVEGF containing blocking antibody (+Ab) as described under "Materials and Methods."

senescent conditioned medium to stimulate HUVEC invasiveness, reducing it to about the level stimulated by presenescent conditioned medium (Fig. 1A). Nonetheless, the antibody failed to inhibit a significant fraction (~45%) of the invasion stimulated by senescent conditioned medium (Fig. 1A). Still, the VEGF-dependent blocked fraction (72 cells/field) triggered by senescent conditioned medium was significantly higher ( $p < 0.02$ ) compared with the VEGF-dependent fraction stimulated by presenescent conditioned medium (45 cells/field). The blocking antibody was very effective (>90%) at blocking the ability of recombinant VEGF to stimulate HUVEC invasiveness (Fig. 1B). For this experiment, recombinant VEGF was used at 15 ng/ml, a concentration exceeding that present in senescent conditioned medium by >7-fold (see Fig. 2). These findings suggest that senescent fibroblasts secrete more VEGF than presenescent fibroblasts but also secrete factors in addition to VEGF that promote HUVEC invasion.

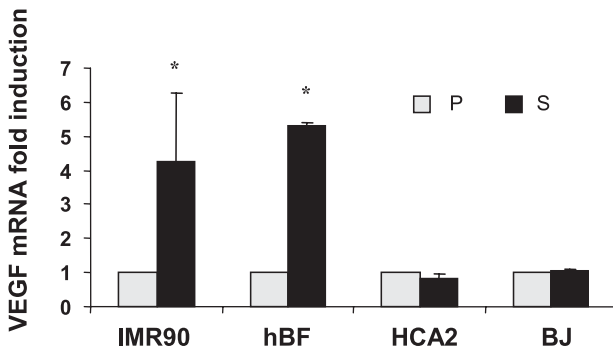
**VEGF is Up-regulated in Senescent Fibroblasts**—To directly determine whether senescent fibroblasts up-regulate VEGF expression, we used real-time quantitative PCR and an ELISA assay to measure VEGF mRNA and secreted protein, respectively. Compared with presenescent fibroblasts, senescent WI-38 fibroblasts expressed 2- to 3-fold more VEGF mRNA (Fig. 2A). Notably, this 2-fold increase in mRNA resulted in a 6-fold increase in secreted VEGF protein (Fig. 2B).

Interestingly, senescent WI-38 fibroblasts expressed no more SDF-1 mRNA or protein than their presenescent counterparts (Fig. 2, C and D). Thus, although senescent fibroblasts have biological properties that overlap with those of tumor-associated fibroblasts (15, 16), in at least some cases senescent





**FIGURE 2. WI-38 senescent fibroblasts express increased levels of VEGF, but not SDF-1 mRNA and protein.** Total RNA and conditioned medium were collected from presenescent (P) or senescent (S) WI-38 fibroblasts. Shown is the average of four independent experiments performed in triplicate  $\pm$  S.D. Asterisks indicate a statistically different change ( $p < 0.05$ ) in expression or secretion compared with P. A, quantitative real-time PCR ratio of total VEGF RNA (all isoforms) relative to the level of 18 S ribosomal RNA. B, secreted VEGF in conditioned medium was determined by ELISA. C, quantitative real-time PCR ratio of total SDF-1 RNA relative to the level of 18 S ribosomal RNA. D, secreted SDF-1 in conditioned medium was determined by ELISA.



**FIGURE 3. VEGF expression in various senescent fibroblast strains.** Total RNA was collected from presenescent (P) or senescent (S) IMR-90, hBF, BJ, and HCA2 fibroblasts, and quantitative real-time PCR was used to determine the ratio of total VEGF RNA (all isoforms) relative to the level of H1a gene. Shown is the average of three independent experiments. Asterisks indicate a statistically different change ( $p < 0.05$ ) in expression compared with P.

and tumor-associated fibroblasts may achieve similar biological effects by different molecular mechanisms.

Analysis of other fibroblast cell strains confirmed that increased VEGF expression was not restricted to WI-38. Indeed, we found 4- to 5-fold more VEGF expression in senescent compared with presenescent human fetal lung IMR90 and human adult breast fibroblasts (Fig. 3, *hBF*). Surprisingly, no increase in VEGF expression was observed in human neonatal foreskin HCA2 and BJ fibroblasts (Fig. 3). The reason why VEGF up-regulation is not a hallmark of all senescent fibroblast cell lines remains to be determined; cell strain-specific differences in p16 regulation may play a role (see "Discussion").

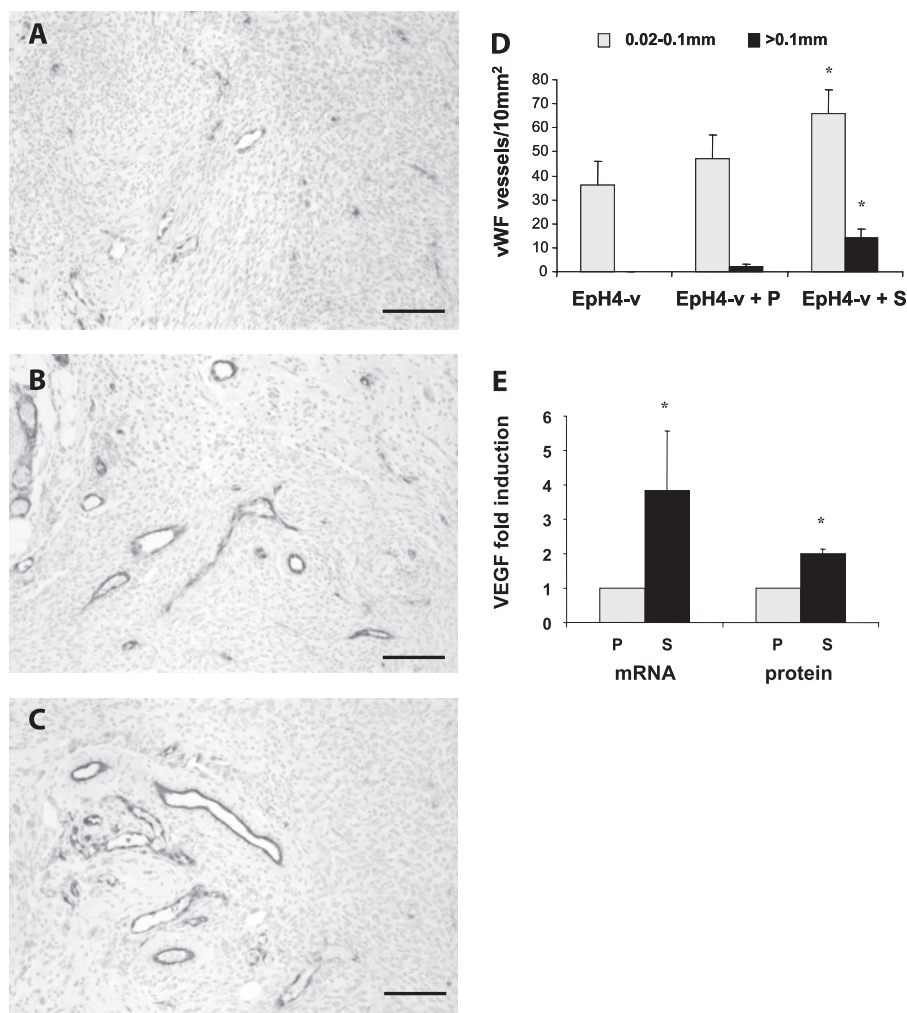
**Senescent Fibroblasts Increase Vascularization of Tumor in Mice**—To determine the physiological relevance of the senescent-associated increase in VEGF expression we injected weakly tumorigenic Eph4-v mouse epithelial cells alone or together with presenescent or senescent mouse breast fibroblasts (mBF). Two months later, the Eph4-v tumors were excised and immunostained for the vWF endothelial-specific marker. The results revealed very significant augmentation in vascularization when senescent mBF were co-injected with Eph4-v (Fig. 4, A–C). Both the number and size of vWF-positive vessels were increased by the co-injection of senescent mBF

(Fig. 4D). Interestingly, the most striking difference was the presence of numerous large vessels ( $>0.1$  mm) in the mBF senescent group. Large vessels were virtually absent from control tumors resulting from Eph4-v cells injected alone or together with presenescent mBF. Of particular interest, increased vascularization was observed despite mBF having only a 2-fold change in VEGF secretion at senescence (Fig. 4E). As expected, tumors excised from animals injected with Eph4-v cells alone or together with presenescent fibroblasts were on average significantly smaller (74 and 105 mm<sup>3</sup>, respectively) compared with

tumors injected with senescent fibroblasts (174 mm<sup>3</sup>). Taken together, these results suggest that one mechanism by which senescent stromal cells facilitate tumor progression is by enhancing blood vessel formation.

**Increased VEGF Expression Is Senescence Specific**—Is the enhanced VEGF expression due to the senescent phenotype *per se* or, alternatively, to the growth-arrested state of senescent fibroblasts? To answer this question, we cultured presenescent fibroblasts in serum-deficient medium for 3 days, which caused them to arrest growth in a reversible quiescent state (data not shown). In contrast to senescence, quiescence failed to increase VEGF mRNA or protein levels (Fig. 5A). These results suggest that enhanced VEGF production is not a consequence of the growth state of senescent cells but rather is a feature of the senescent phenotype.

To more critically test the idea that enhanced VEGF expression is a consequence of senescence, we induced presenescent WI-38 cells to undergo rapid senescence by ectopically expressing p16<sup>INK4a</sup> or oncogenic RAS (Ha-RAS<sup>V12</sup>), two known inducers of a rapid senescence response (23, 27, 28). We expressed p16<sup>INK4a</sup>, oncogenic RAS, or a control protein (green fluorescent protein, GFP) using lentiviral vectors, which allowed rapid ectopic expression in  $>99\%$  of the population (see "Materials and Methods"). p16<sup>INK4a</sup> expression reduced cell proliferation within 4 days, whereas oncogenic RAS slightly stimulated proliferation before arresting growth, as expected (23, 27, 28). In either case, within 7 days after lentiviral transduction, cells ceased proliferation (Fig. 5B) and developed a senescent phenotype as determined by their large flat morphology and expression of the senescence-associated  $\beta$ -galactosidase (not shown) as described (2). By contrast, cells infected with the control GFP-expressing lentivirus continued to proliferate (Fig. 5B). Quantitative real-time PCR and ELISA assays showed that VEGF mRNA and secreted protein levels increased in response to p16<sup>INK4a</sup> and oncogenic RAS, but not GFP (Fig. 5, C and D), to approximately the same levels induced by replicative senescence. Thus, WI-38 fibroblasts responded to replicative exhaustion, p16<sup>INK4a</sup> expression, and expression of oncogenic RAS, all of which induce senescence, by up-regulating VEGF expression.



**FIGURE 4. Increased tumor vascularization by senescent fibroblasts.** Murine EpH4-v epithelial cells were injected alone or in combination with presenescent (P) or senescent (S) mouse breast fibroblasts into *nu/nu* mice (A, B, and C, respectively). After two months, tumors formed at all injection sites (three tumors from each group) were analyzed. A–C, vWF immunostaining of paraffin-embedded tumor sections. A representative picture from each group is shown. Scale bars, 0.1 mm. D, quantification of vWF-positive vessels from each tumor group ( $n = 3$ ). Vessel counts were separated into two groups, small (0.02–0.1 mm) and large (>0.1 mm). E, mouse breast fibroblast total VEGF RNA and secreted VEGF levels in conditioned medium were determined by quantitative real-time PCR and ELISA, respectively. Asterisks indicate a statistically different change ( $p < 0.05$ ) in expression or secretion compared with P.

**Senescent Fibroblasts Retain VEGF Inducibility by Hypoxia—** The best-studied inducer of VEGF expression is hypoxia, which increases VEGF transcription by stabilizing and activating the hypoxic-inducible (transcription) factor 1  $\alpha$  (HIF-1 $\alpha$ ) (18, 29). To determine whether the senescence-induced increase in VEGF production is caused by the same mechanism used by hypoxia, we determined the levels of HIF-1 $\alpha$  in presenescent and senescent WI-38 fibroblasts. Senescent cells expressed only slightly more (<2-fold) HIF-1 $\alpha$  than presenescent cells, a level far less than that induced by hypoxia (Fig. 6A). This was true whether cells were made senescent by replicative exhaustion or overexpression of p16<sup>INK4a</sup> (Fig. 6A). Interestingly, although hypoxia caused a striking accumulation of HIF-1 $\alpha$  in presenescent cells, as expected, hypoxia failed to increase HIF-1 $\alpha$  levels in senescent cells (Fig. 6A). These findings suggest that the classic mechanisms responsible for the hypoxic response may be impaired in senescent cells. Despite impaired hypoxia-induced

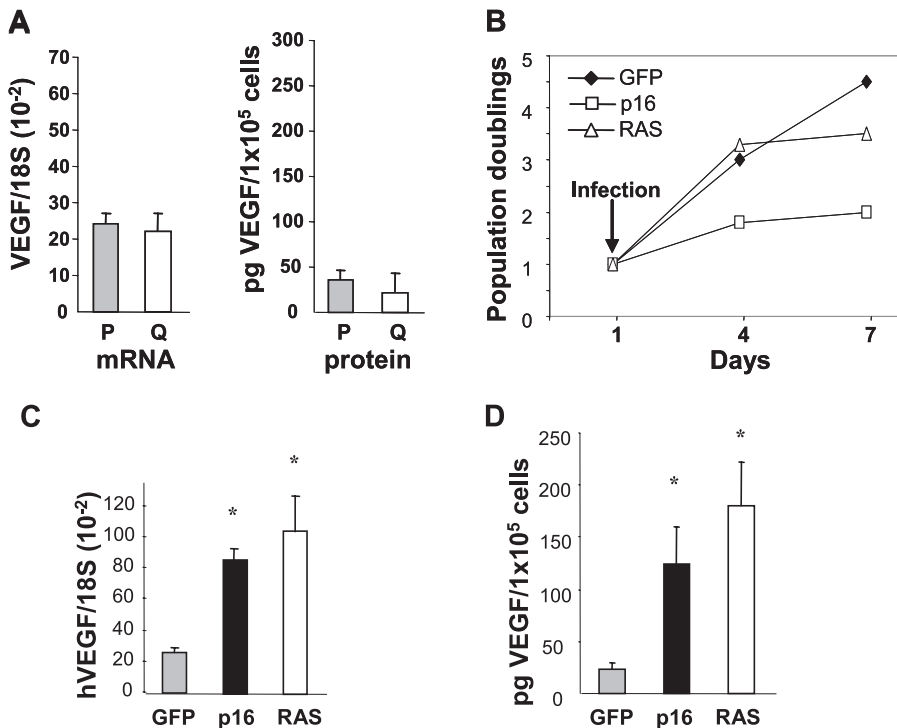
HIF-1 $\alpha$  expression, senescent cells up-regulated VEGF mRNA and protein secretion in response to hypoxia (Fig. 6B). Of note, the amount of VEGF secreted by senescent cells solely in response to hypoxia (~1000 pg) exceeded that secreted by presenescent cells solely in response to hypoxia (~400 pg) (compare Fig. 2 with Fig. 6). These results suggest that senescence may synergize with hypoxia to stimulate VEGF production.

## DISCUSSION

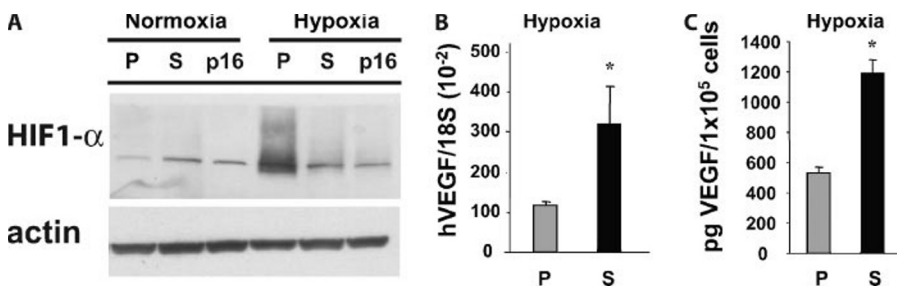
Senescence, like apoptosis, most likely evolved to prevent cancer by arresting the growth of cells in danger of becoming neoplastic. However, senescent cells may also contribute to aging and age-related disease, including late life cancer, consistent with the senescence response being an example of antagonistic pleiotropy (6). The deleterious effects of senescent cells are thought to derive from the myriad factors they secrete. These factors include matrix-degrading proteases, growth factors, and inflammatory cytokines (6, 7, 30), all of which can disrupt normal tissue homeostasis and favor cancer development. Here, we have shown that most, but not all, human and mouse fibroblast strains secrete biologically active VEGF at senescence. VEGF is a potent angiogenic factor known to be important for cancer progression (31).

We previously showed that senescent WI-38 fibroblasts promote the conversion of preneoplastic epithelial cells to malignant tumors when co-injected into immunocompromised mice (10). Here we have shown that tumors that develop in the presence of senescent fibroblasts are substantially more vascularized than tumors that develop in the presence of presenescent fibroblasts or in the absence of fibroblasts. Strikingly, senescent cells not only augmented the number of blood vessels/mm<sup>2</sup> but increased their size as well. In fact, we routinely found large vessels (defined as >0.1 mm) only in tumors that developed in the presence of senescent fibroblasts. These results suggest that the presence of senescent fibroblasts may have favored tumor vascularization early on.

Invasion of HUVEC cells is a prerequisite for angiogenesis. Accordingly, we found that some senescent cells, by secreting VEGF and other important factors, stimulated HUVEC to migrate and invade a basement membrane in culture. Indeed, invasion of HUVEC in the presence of a VEGF-blocking anti-



**FIGURE 5. Increased VEGF production at senescence is specific and independent of the senescence inducer.** Total RNA and conditioned medium were collected from presenescent (P) or quiescent (Q) WI-38 fibroblasts. *A*, total VEGF RNA (all isoforms) relative to the level of 18 S ribosomal RNA and secreted VEGF in conditioned medium were determined by quantitative real-time PCR and ELISA, respectively. *B*, presenescent WI-38 fibroblasts at population doubling 28 were infected on day 1 with lentiviruses expressing enhanced GFP (GFP), p16<sup>INK4a</sup> (p16), or oncogenic Ha-RAS<sup>V12</sup> (RAS) and total cell number determined on days 4 and 7 thereafter. p16- and RAS-expressing cells had ceased growth by day 4 after the infection, whereas GFP-expressing cells continued proliferation through day 7. *C*, total RNA was isolated from the indicated infected populations 4 days after infection and VEGF mRNA determined as in panel *A*. *D*, conditioned medium was collected for 48 h 4 days after infection and VEGF levels determined by ELISA. Shown is the average of four independent experiments performed in triplicate  $\pm$  S.D. Asterisks indicate a statistically different change ( $p < 0.05$ ) in expression compared with GFP.



**FIGURE 6. Hypoxia increases VEGF independent of HIF-1 $\alpha$  in senescent cells.** Presenescent (P) or senescent (S) WI-38 fibroblasts or presenescent WI-38 cells induced to senescence by p16<sup>INK4a</sup> expression were cultured in atmospheric (normoxia) or reduced (1%; hypoxia) oxygen for 48 h. Shown is the average of three independent experiments performed in triplicate  $\pm$  S.D. Asterisks indicate a statistically different change as determined by a Student *t* test ( $p < 0.05$ ). *A*, HIF-1 $\alpha$  protein levels were determined by Western analysis using actin as a control. *B*, VEGF mRNA levels were determined by quantitative real-time PCR by the ratio of total VEGF RNA (all isoforms) to 18 S ribosomal RNA. *C*, secreted VEGF levels in conditioned medium were determined by ELISA.

body suggested that senescent fibroblasts likely secrete additional angiogenic factors. Surprisingly, SDF-1, an angiogenic factor produced by tumor-associated fibroblasts (16), was not among the factors secreted by senescent WI-38 cells. Thus, although senescent and tumor-associated fibroblasts have overlapping biological effects on neighboring preneoplastic or neoplastic epithelial cells (10, 15, 16), they may use distinct mechanisms to achieve at least some of their biological effects.

Quiescent WI-38 cells did not up-regulate VEGF, suggesting that elevated VEGF secretion is a senescence-associated, rather

than simply growth state-associated, phenotype. Quiescence is a reversible arrested state that results in part from increased expression of p21<sup>CIP1</sup>, a cyclin-dependent kinase inhibitor and p53 transcriptional target (32, 33). p53 and p21<sup>CIP1</sup> are also important for the essentially irreversible arrest of senescent cells (32, 34). In some senescent cells, including WI-38 fibroblasts, the irreversibility of the arrest is due in large measure to increased expression of a different cyclin-dependent kinase inhibitor, p16<sup>INK4a</sup> (23, 35). Not all human fibroblast strains senesce with elevated p16<sup>INK4a</sup> levels. For example, human BJ and HCA2 foreskin fibroblasts senesce with high levels of p21<sup>CIP1</sup> but undetectable levels of p16<sup>INK4a</sup>, and senescence in these cells, but not WI-38, is reversible upon inactivation of p53 (23). It is interesting, then, that VEGF levels were not elevated in senescent BJ and HCA2 cells (Fig. 3). This finding suggests that VEGF up-regulation is not common to all senescent fibroblasts and might depend on whether the p16<sup>INK4a</sup>/pRB pathway is engaged at senescence. Consistent with this idea, early passage WI-38 increased VEGF secretion following ectopic overexpression of p16<sup>INK4a</sup> or oncogenic RAS, which requires p16<sup>INK4a</sup> for its ability to induce senescence (36).

We also found that the senescence-associated increase in VEGF production was not accompanied by a commensurate increase in HIF-1 $\alpha$ , the major transcription factor that up-regulates VEGF in response to hypoxia (25). This finding suggests that VEGF up-regulation by senescent cells is not due to classic hypoxic response mechanisms.

Moreover, under hypoxic conditions, we found that senescent cells failed to induce HIF-1 $\alpha$ , in contrast to presenescent cells, which induced HIF-1 $\alpha$  to high levels. Nonetheless, senescent cells up-regulated VEGF in response to hypoxia, and this response synergized with the senescent-associated increase in VEGF secretion. At present, we do not know whether the slightly increased basal levels of HIF-1 $\alpha$  in normoxia is responsible for the senescence-associated increase in VEGF or whether other mechanisms, such as diminished expression of Von Hippel-Lindau, or direct activation of HIF-1 $\alpha$  by pRB (37,



38), are responsible. Whatever the mechanism by which senescent cells up-regulate VEGF, our findings provide an additional and potentially important mechanism by which senescent human stromal cells might stimulate cancer progression, namely, up-regulation of angiogenic factors such as VEGF.

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