

# FGF-1–Dependent Proliferative and Migratory Responses Are Impaired in Senescent Human Umbilical Vein Endothelial Cells and Correlate with the Inability to Signal Tyrosine Phosphorylation of Fibroblast Growth Factor Receptor-1 Substrates

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**Abstract.** Senescent cells do not proliferate in response to exogenous growth factors, yet the number and affinity of growth factor receptors on the cell surface appear to be similar to presenescent cell populations. To determine whether a defect in receptor signaling exists, we analyzed human umbilical vein endothelial cells (HUVEC) since HUVEC growth is absolutely dependent upon the presence of FGF. We report that in both presenescent and senescent HUVEC populations, FGF-1 induces the expression of cell cycle–specific genes, suggesting that functional FGF receptor (FGFR) may exist on the surface of these cells. However, the tyrosine phosphorylation of FGFR-1 substrates, Src and cortac-

tin, is impaired in senescent HUVEC, and only the presenescent cell populations exhibit a FGF-1–dependent Src tyrosine kinase activity. Moreover, we demonstrate that senescent HUVEC are unable to migrate in response to FGF-1, and these data correlate with an altered organization of focal adhesion sites. These data suggest that the induction of gene expression is insufficient to promote a proliferative or migratory phenotype in senescent HUVEC and that the attenuation of the FGFR-1 signal transduction pathway may be involved in the inability of senescent HUVEC to proliferate and/or migrate.

**E**ARLY studies of cellular senescence have demonstrated that the *in vitro* lifespan of human diploid fibroblasts is finite and is a function of the cumulative population doublings (17, 35, 47). These phenomena have been associated with different cell types, and the human endothelial cell presents an interesting alternative for the study of cellular senescence *in vitro*. Unlike the fibroblast, the human umbilical vein endothelial cell (HUVEC)<sup>1</sup> is absolutely dependent upon the presence of FGF for *in vitro* proliferation and serial propagation (26). It is well established that senescent HUVEC are refractory to the mitogenic potential of FGF (46). Further, it has been demonstrated that although FGF is a potent mitogen for HUVEC, its function is antagonized in the presence of the cytokine interleukin (IL)-1 (6, 30), and IL-1 $\alpha$  may act *in*

tracellularly to inhibit proliferation (14). Indeed, this laboratory has shown that an exaggerated level of intracellular IL-1 $\alpha$  exists in senescent HUVEC (15), which may function to repress FGF-1–induced HUVEC growth in senescent populations (27).

The fibroblast growth factor receptor (FGFR) signaling pathway has been defined (12) and includes the tyrosine kinase activation of the FGFR and subsequent phosphorylation of a variety of intracellular substrates, including Src (51, 53). Src, a member of a family of cytosolic tyrosine kinases, is thought to be involved in signal transduction events underlying growth control (5). The protein kinase activity of Src is negatively regulated when tyrosine 527 is phosphorylated by another tyrosine kinase, Csk (28, 31, 33, 39). It has been suggested that Csk mediates the activity of Src through its own SH2 and SH3 domains, and these are required to target Csk to sites where Src is active (18). In addition, the phosphorylation of Src Y527 as well as the SH2 and SH3 domains in the amino terminus of Src have been identified as important regulatory regions responsible for the redistribution of Src from cellular membranes to focal adhesion sites (21, 22). Because several Src substrates are localized to focal adhesion sites (41), the redistribution of activated Src suggests it may play a role in the regulation of cell adhesion. Indeed, it has recently been shown that Src can function during the early stages of fibronectin-mediated cellular adhesion of

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1. *Abbreviations used in this paper:* chx, cycloheximide; FAS, focal adhesion sites; FGFR, FGF receptor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; H3, histone 3.2; HUVEC, human umbilical vein endothelial cell; IL, interleukin; IME, immediate-early; IRAP, IL-1 receptor antagonist; MTL, middle-to-late; ODC, ornithine decarboxylase; RT-PCR, reverse transcription polymerase chain reaction.

fibroblasts (23). Further, although focal adhesions have been shown to be associated with stationary cells, they also will form at the leading edge of motile cells (3), suggesting that Src may also function in the regulation of cell migration, and an increased tyrosine phosphorylation in the focal adhesions of migrating HUVEC has been observed (38). In addition, a Src-dependent increase in tyrosine phosphorylation of several cytoskeletal proteins, including cortactin (45), has also been demonstrated. Cortactin is a filamentous (F)-actin binding protein that contains an SH3 domain and can be phosphorylated on both serine/threonine and tyrosine residues (49, 50). Our laboratory identified murine cortactin as a substrate for Src and demonstrated an association between cortactin and Src during the entire G<sub>1</sub> phase of cell cycle after activation of Balb/c 3T3 cells by FGF-1 (52, 53). Thus, the FGF-1 signal transduction pathway appears to include the association and phosphorylation of FGFR-1 and Src and the association of Src with cortactin, and these events may affect cellular proliferation, migration, and cytoskeletal properties.

Although senescent cells do not proliferate in response to exogenous growth factors, the number and affinity of growth factor receptors appear to be similar in presenescent and senescent populations (7, 10, 36, 43). However, in senescent WI-38 fibroblasts, Fos transcription in response to serum (42) and the protein tyrosine kinase activity of the epidermal growth factor receptor (EGFR) (4) have been shown to be compromised, suggesting that a defect in receptor signaling may exist. To determine the nature of such a defect, we analyzed HUVEC populations since HUVEC growth is absolutely dependent upon the presence of exogenous FGF (26). We observed an induction of cell cycle-specific genes in response to FGF-1 in both early and late passage cells, suggesting that a functional FGFR may be present on the surface of these cells. However, analysis of the ability of the FGFR to induce the tyrosine phosphorylation of its substrates revealed that although presenescent HUVEC populations were able to induce the tyrosine phosphorylation of Src and cortactin in response to FGF-1, senescent HUVEC were unable to do so. Further, the presenescent HUVEC exhibited a FGF-1-dependent Src tyrosine kinase activity, but senescent cells revealed a baseline level of kinase activity that was independent of the presence of FGF-1. These data suggest that the inability of senescent HUVEC to respond to FGF-1 may involve a defect downstream and/or independent of receptor binding and activation of gene expression. Further, we were able to demonstrate that senescent HUVEC were unable to migrate in response to FGF-1, which correlated with prominent focal adhesion sites. Thus, despite the ability of the FGFR to signal gene expression in senescent HUVEC, the FGF-1-dependent proliferative and migratory responses appear to be compromised, and these responses may involve the inability of the cells to use the FGFR pathway to signal the tyrosine phosphorylation of Src and the inability of Src to phosphorylate cortactin.

## Materials and Methods

### Cell Culture

HUVECs, strain H3605, used in these experiments were a generous gift

from M. Gimbrone (Harvard Medical School, Boston, MA). HUVECs were serially passaged at 1:5 split ratios in medium 199 (RHJ Biosciences, Lenexa, KS) supplemented with 10% (vol/vol) FBS, heparin (Upjohn, Kalamazoo, MI; 5 U/ml), 1× antibiotic-antimycotic (GIBCO, BRL, Gaithersburg, MD), and crude bovine FGF-1 (150 µg/ml) in cell culture dishes coated with fibronectin (5 µg/cm<sup>2</sup>). Human fibronectin and crude preparations of bovine FGF-1 were prepared as previously described (26). At each passage, the cells were trypsinized when confluent, counted in a hemacytometer, and seeded at known cell densities. The population doubling level was calculated as described (26). Cells with <25 cumulative population doublings (CPDs) were considered presenescent and cells with more than 60 CPDs were considered senescent. The senescent cells used in these experiments completed 90–95% of their lifespan and were maintained in culture for at least 7 d without an increase in cell number. Further, *in situ* labeling experiments were performed according to the methods of Stein et al. (44). To determine the labeling index, [<sup>3</sup>H]thymidine-positive nuclei were counted in 10 or 20 fields in presenescent or senescent cells, respectively. The presenescent HUVEC (CPD 24) had 85% of the nuclei labeled, whereas in the near-senescent populations (CPD 65), only 8% of the nuclei were labeled.

### RNA Extraction, Northern Blot Analysis, and Reverse Transcriptase-Polymerase Chain Reaction

Total RNA was extracted from HUVEC monolayers by the guanidinium isothiocyanate procedure as described (14). RNA (10 µg) was separated on 0.8% agarose gels and capillary-blotted onto Zeta-Probe nylon membranes (BioRad Labs, Hercules, CA). <sup>32</sup>P-labeled cDNA probes were hybridized to the RNA at 65°C for 18 h. The membranes were washed at high stringency and exposed on Kodak X-AR film (Rochester, NY) at -80°C. The Fos and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNAs were purchased from the American Type Culture Collection (Rockville, MD). The histone 3.2 (H3) cDNA was a generous gift from J. Campisi (Berkeley, CA), and the human ornithine decarboxylase (ODC) cDNA was provided by J.K. deRiel (Temple University, Philadelphia, PA).

Reverse transcription polymerase chain reaction (RT-PCR) was performed using 1 µg total RNA as described (14). The cDNA was diluted with H<sub>2</sub>O to 500 µl and PCR was performed with 5 µl of each sample amplified by denaturation at 94°C for 1 min, annealed at 54°C for 2 min, and elongated at 72°C for 3 min using 35 cycles for the FGFR family and 32 cycles for GAPDH. Specific primer sequences for each gene were as follows: FGFR-1: sense: 5'-AAGGACAAACCCAACCGTGTGACC-3', antisense: 5'-CCCAAAGTCTGCTATCTTCATCAC-3'; FGFR-2: sense: 5'-CTGTGCCGAATGAAGAACACGACC-3', antisense: 5'-CCCAAAGTCTGCTATCTTCATCAC-3'; FGFR-3: sense: 5'-TGAGGACACAGGTGTGGACACAGG-3', antisense: 5'-TCTGCCGGATGCTGCCAA ACTTGT-3'; FGFR-4: sense: 5'-CTGCCCGCCTCGTGAGTCTAGAT-3', antisense: 5'-CCCAAAGTCTGCTATCTTCATCAC-3'; GAPDH: sense: 5'-CCCAATGGCAAATCCATGGCA-3', antisense: 5'-TCT AGACGGCA-GGTCAGGTCCACC-3'.

### Immunoprecipitation and Western Blot or In Vitro Kinase Analysis

Presenescent and senescent populations of HUVEC were incubated for 7.5 h in media lacking FGF-1 and heparin and containing 3% FBS and 20 ng/ml PMA. The cells were washed twice with PBS and incubated for a total of 24 or 48 h in media containing 1.5% FBS, FGF-1 (10 ng/ml) and heparin (10 µg/ml) were added for the indicated times and the cells were scraped into 1 ml PBS and collected by centrifugation (5 s). The cell pellets were lysed in 1 ml RIPA (10 mM Tris-HCl, pH7.5, 1 mM EDTA, 150 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 1% Triton X-100) containing 1 mM PMSF, 2 µg/ml aprotinin, 10 µg/ml leupeptin, and 1 mM Na<sub>3</sub>VO<sub>4</sub> and incubated on ice for 5 min. The cell lysates were centrifuged at 4°C for 10 min and the supernatants were transferred to fresh tubes. Specific antibodies were incubated with cell lysates and rotated at 4°C for 1 h followed by the addition of 20 µl Protein A-Sepharose (Pharmacia LKB Biotechnology Inc., Piscataway, NJ) and rotation at 4°C for 1 h. The immunoprecipitates were washed three times with RIPA buffer, dissolved in SDS-sample buffer (125 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 0.001% bromophenol blue), and resolved by SDS-PAGE using 7.5% (wt/vol) acrylamide gels. The proteins were transferred to 0.2 µm nitrocellulose membranes, and Western blot analysis was performed with specific antibodies in Tris-buffered saline (20 mM Tris-HCl, pH 7.6, 137 mM NaCl, 0.1% Tween-20) containing either 5% milk (Src-2 blots) or 5%

BSA (phosphotyrosine blots) using enhanced chemiluminescence (Amersham Corp., Arlington Heights, IL) according to the manufacturer's instructions.

To analyze the tyrosine kinase activity of Src, *in vitro* kinase assays were performed. The cells were lysed and immunoprecipitated with Src-specific antibodies as described above, washed with kinase buffer (30 mM Tris-HCl, pH 7.4, 10 mM MnCl<sub>2</sub>), and then incubated in 50  $\mu$ l kinase buffer containing 2  $\mu$ g acid-denatured enolase (Sigma Immunochemicals, St. Louis, MO) and 10  $\mu$ Ci  $\gamma$ [<sup>32</sup>P]ATP for 10 min at room temperature. The phosphorylated proteins were visualized by autoradiography of dried 10% (wt/vol) polyacrylamide gels following SDS-PAGE.

### Antibodies

The antibodies for immunoblot analysis included: (a) polyclonal rabbit anti-human Src (N-16; Santa Cruz Biotechnology, Santa Cruz, CA), an antibody raised against a peptide corresponding to the unique residues 3–18 within the amino terminus of the human Src gene and used for Src immunoprecipitation; (b) Src-2 (Santa Cruz Biotechnology), a polyclonal antibody raised against a peptide corresponding to the carboxyl terminus of Src and cross-reactive with Yes and Fyn and used for Src Western blot analysis; (c) monoclonal antiphosphotyrosine (Upstate Biotechnology Inc., Lake Placid, NY) used for Western blot analysis; (d) polyclonal rabbit anti-mouse cortactin antisera 2719, generated from a peptide antigen (residues 343–362 of murine cortactin) injected into rabbits and used for immunoprecipitation of cortactin protein (52); and (e) anticortactin antisera 001, generated from recombinant murine cortactin expressed as a glutathione S-transferase fusion protein and used for Western blot analysis of cortactin (53).

### HUVEC Migration Assays

Monolayers of early and late passage HUVEC were wounded as previously described (40). Briefly, confluent monolayers were scraped with a razor blade and cellular debris was removed by washing with PBS. The monolayers were incubated for 16–20 h at 37°C in medium 199 containing 10% FBS alone (control) or supplemented with FGF-1 (10 ng/ml), heparin (10  $\mu$ g/ml), rIL-1 $\alpha$  (10 ng/ml), IL-1 receptor antagonist IRAP; (100 ng/ml), or PMA (20 ng/ml) as indicated in the text. The cells were then fixed in 25% acetic acid/75% methanol and stained with hematoxylin (Sigma Immunochemicals). The number of cells migrating from the wound origin was counted with a light microscope at 100 magnification using a grid. The values represent the mean of five fields, and three separate experiments have been performed with each condition tested in duplicate.

### Immunofluorescence

Presenescent and senescent HUVEC were plated on glass coverslips coated with fibronectin, incubated for 24 h in medium 199 containing 10% FBS, 1 $\times$  antibiotic-antimycotic, 10 ng/ml FGF-1 and 10  $\mu$ g/ml heparin, and then fixed in ice-cold acetone. The coverslips were dried, incubated in immunofluorescence-blocking buffer (PBS containing 5% bovine serum albumin, 0.1% Triton-X 100, 0.1% Tween-20, 0.1% NaN<sub>3</sub>) for 1 h, washed three times with PBS, and incubated 1 h with antibody diluted 1:50 in PBS (monoclonal antivinculin antibody [Sigma Immunochemicals]; monoclonal antiphosphotyrosine antibody [Upstate Biotechnology Inc.]; and rabbit antiserum for the fibronectin receptor [ $\alpha$ 5 $\beta$ 1], a gift from S. Argraves, Holland Laboratory). The coverslips were washed three times in PBS, incubated 30 min in fluorescein-conjugated goat anti-mouse or anti-rabbit antibodies, washed three times in PBS, and embedded in 50% glycerol with 0.1% phenylenediamine. The cells were photographed with a fluorescence microscope at 600 magnification (Olympus Corp., Lake Success, NY).

## Results

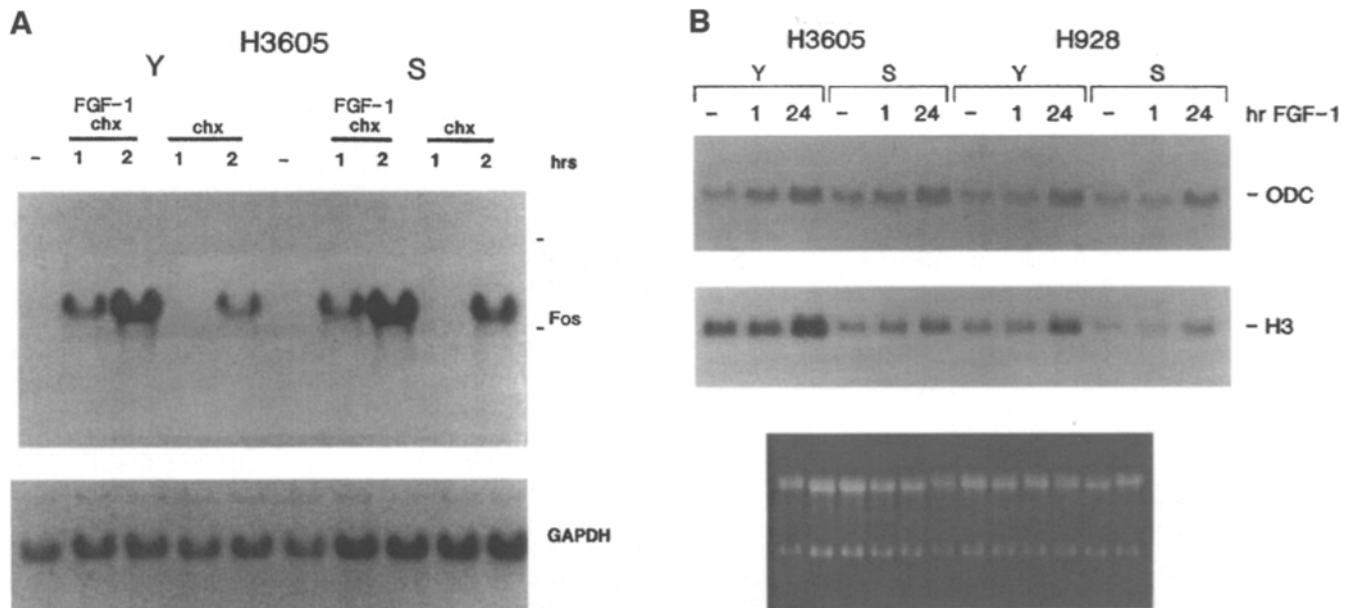
### Expression of Cell Cycle-Specific Genes in Presenescent and Senescent HUVEC

It has been suggested that a defect in receptor signaling may exist in senescent cell populations (4). Because HUVEC are absolutely dependent upon the presence of FGF-1 for proliferation, we have studied the FGF-1-signaling

pathway in senescent HUVEC to better understand the processes that may be involved in regulating cellular senescence *in vitro*. There are currently four FGFR genes that have been identified (12). RT-PCR analysis of the steady state level of the FGFR gene family demonstrated that the primary gene, which is expressed at similar levels in both presenescent and senescent HUVEC populations, is the FGFR-1 transcript (Fig. 1 C). The total number of FGFR-1 on the surface of HUVEC is extremely low (13) and is not detectable using standard methods of FGFR immunoprecipitation followed by either immunoblot analysis or by an *in vitro* kinase assay (data not shown). Thus, we have chosen to study the functional aspects of FGFR-1 signaling through the expression of immediate-early (IME) and mid-to-late (MTL) cell cycle-dependent genes. This approach was taken because cell cycle-specific gene expression has been used as a marker for the progression of cells through the G<sub>1</sub> and S phases in response to growth factors (32, 42). Early and late passage HUVEC were made quiescent in media lacking FGF-1 and heparin, and then treated with FGF-1 for the indicated times (Fig. 1). Northern blot analysis revealed that both HUVEC populations treated with FGF-1 and cycloheximide (chx) demonstrated a superinduction of the IME gene, Fos, as compared to the induction with chx alone (Fig. 1 A). Because Northern blots are not as sensitive as PCR, we used chx to visualize the very low level of Fos mRNA in HUVEC (Fig. 1 A); however, these results are consistent with RT-PCR in which an induction of Fos mRNA was also observed in presenescent and senescent populations treated with FGF-1 alone (data not shown). In addition, we analyzed the mRNA expression of the MTL genes, ODC and H3, and found that ODC was induced in response to FGF-1 in two different strains of early and late passage HUVEC (Fig. 1 B). Further, although the steady state level of H3 mRNA appears to be decreased in the senescent HUVEC populations, a FGF-1 induction was observed in the presenescent and senescent cells from both strains (Fig. 1 B). Thus, it appears that FGF-1 is capable of signaling an induction of gene expression through the FGFR-1 on the surface of presenescent, as well as senescent, HUVEC populations.

### The FGF-1-dependent Tyrosine Phosphorylation of FGFR-1 Substrates and FGF-1-dependent Tyrosine Kinase Activity of Src Is Impaired in Senescent HUVEC

The results described above imply that the functional properties of the FGFR-1 in senescent HUVEC are not impaired. However, FGF-1 mutants and FGF-1 chimera proteins have shown that FGFR-binding activity and protooncogene expression can occur without mitogenic activity (2, 20) and FGF-1-induced DNA synthesis may occur without increases in the level of tyrosine phosphorylation (48). Therefore, because the dissociation of cellular events has been described, we questioned whether FGFR-1 signaling was functional for the tyrosine phosphorylation of receptor substrates in presenescent and senescent HUVEC. Our laboratory previously identified two predominant tyrosine phosphorylated proteins in FGF-1-treated Balb/c 3T3 cells during the MTL G<sub>1</sub> phase of the cell cycle with molecular weights of 80 and 60 kD, corresponding to

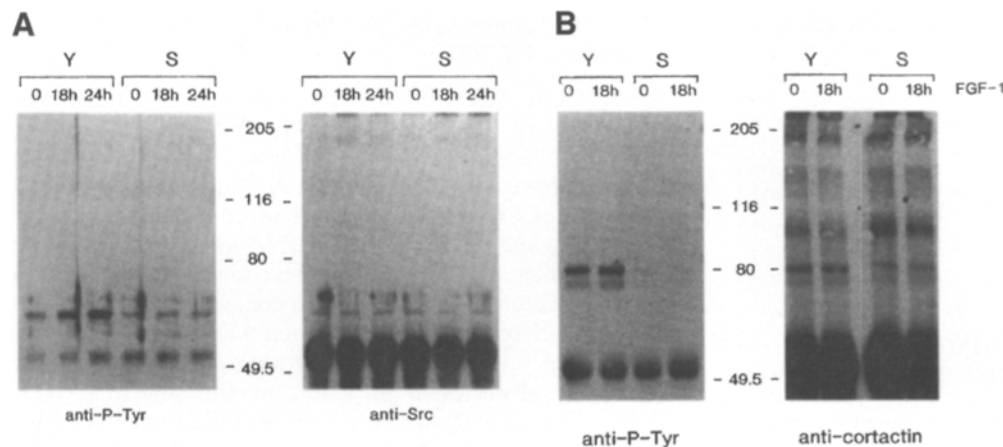


**Figure 1.** Expression of cell cycle-specific genes in presenescent and senescent populations of HUVEC. (A) Presenescent (young; Y) and senescent (S) HUVEC monolayers of strain H3605 were starved for 48 h in media lacking FGF-1, and then stimulated for 1 or 2 h with FGF-1 (10 ng/ml), heparin (10  $\mu$ g/ml) and cycloheximide (chx; 10  $\mu$ g/ml), or chx only, as indicated. Total RNA was purified and 10  $\mu$ g was separated on agarose gels for Northern blot analysis using a  $^{32}$ P-labeled cDNA probe specific for Fos. The filter was stripped and rehybridized to a radiolabeled GAPDH cDNA probe to confirm that each sample contained similar RNA levels. (B) Presenescent (young; Y) and senescent (S) cells from HUVEC strains H3605 and H928 were starved for 48 h and then stimulated

with FGF-1 (10 ng/ml) and heparin (10  $\mu$ g/ml) for 1 or 24 h. Total RNA was purified and 10  $\mu$ g was separated on agarose gels for Northern blot analysis using ornithine decarboxylase (ODC) or histone 3.2 (H3)  $^{32}$ P-labeled cDNA probes. The gel was stained with ethidium bromide to determine the amount of RNA per lane. (C) Total RNA was purified from presenescent (young; Y) and senescent (S) HUVEC monolayers (strain H3605) and used for RT-PCR with sense and antisense primers for the FGFR gene family and GAPDH (see Materials and Methods). The products of amplification were separated on a 1% agarose gel and stained with ethidium bromide.

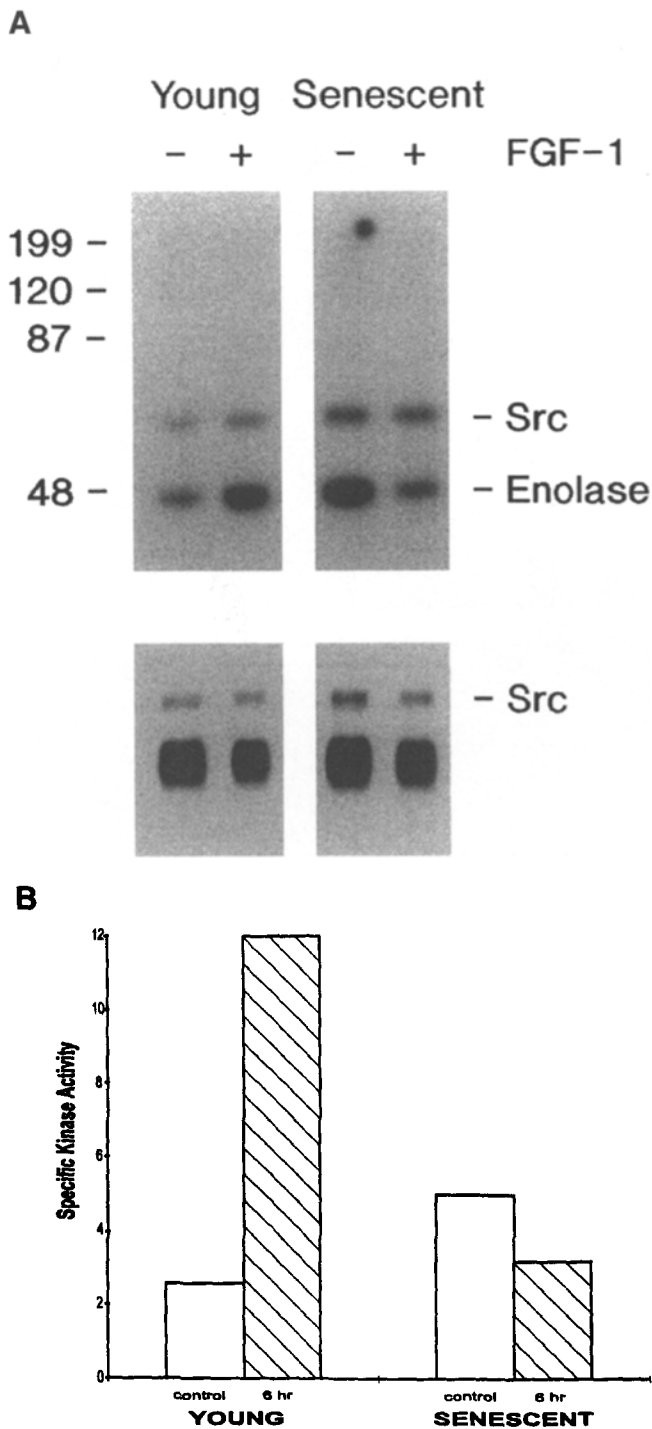
murine cortactin and Src, respectively (51–53). Immunoprecipitation of presenescent HUVEC populations with a Src-specific antibody followed by antiphosphotyrosine immunoblot analysis showed a FGF-1-dependent induction of Src tyrosine phosphorylation (Fig. 2 A). Interestingly, in several experiments with early passage HUVEC, Src poorly induced the tyrosine phosphorylation of cortactin in response to FGF-1 (Fig. 2 B). In contrast, senescent

HUVEC populations were impaired in their ability to induce the tyrosine phosphorylation of Src in response to FGF-1 and displayed little if any tyrosine phosphorylated cortactin, despite similar protein levels in both presenescent and senescent populations (Fig. 2). To quantitate these results, the autoradiographs were evaluated by densitometric scanning, dividing the intensity of the phosphorylated Src or cortactin band by the appropriate protein



**Figure 2.** The FGF-1-induced tyrosine phosphorylation of Src and cortactin is impaired in senescent HUVEC. Presenescent (young; Y) and senescent (S) HUVEC from strain H3605 were starved for a total of 24 h and then treated with FGF-1 and heparin for 18 or 24 h as described in Materials and Methods. The cells were collected, lysed, and immunoprecipitated with antibodies specific for (A) Src, N-16, or (B) cortactin, #2719, as described in Materials and

Methods. The immunoprecipitates were resolved by SDS-PAGE using 7.5% polyacrylamide gels, and Western blot analysis was performed using antiphosphotyrosine antibodies (*anti-P-Tyr*), Src antibodies (*anti-Src*), or cortactin antibodies (*anticortactin*).



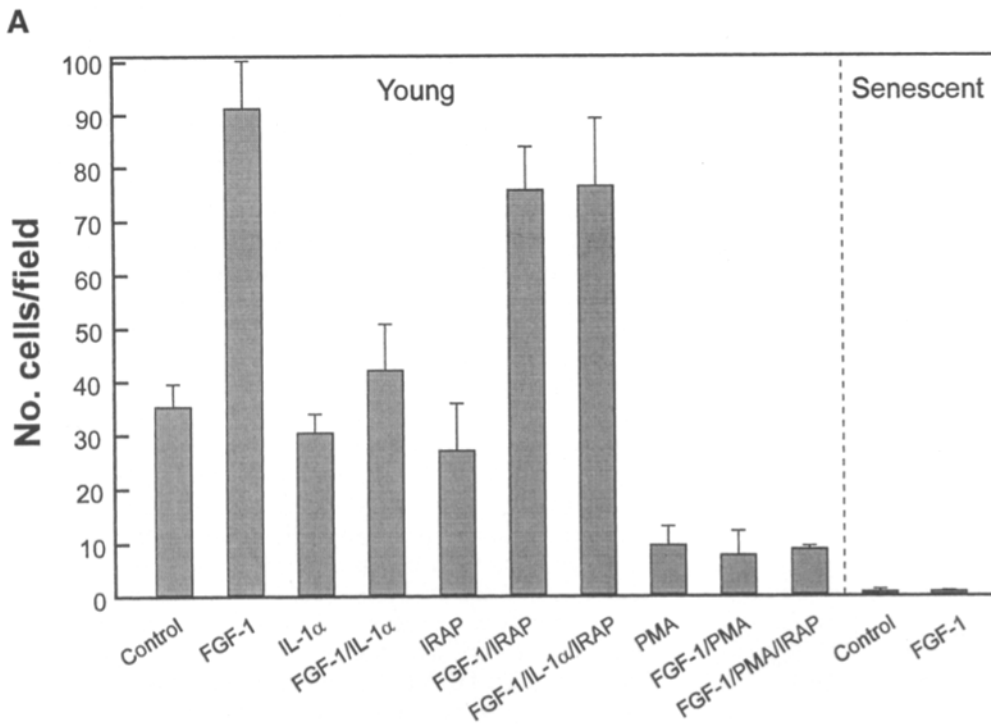
**Figure 3.** The tyrosine kinase activity of Src is impaired in senescent HUVEC. (A) Presenescent (young) and senescent HUVEC from strain H3605 were starved for a total of 48 h and then treated with FGF-1 and heparin for 6 h as described in Materials and Methods. The cells were collected, lysed, and immunoprecipitated with an antibody specific for Src, and an in vitro kinase assay was performed using the substrate enolase. After SDS-PAGE, phosphorylated Src and enolase were visualized by autoradiography (*upper*), and the level of Src protein was visualized by Western blot analysis with anti-Src antibodies (*lower*). (B) The specific Src kinase activity was calculated by dividing the intensity of the enolase band (Fig. 3 A, *upper*) by the intensity of the Src protein band (Fig. 3 A, *lower*), as determined by densitometric scanning (Visage version 4.6P; BioImage, Ann Arbor, MI).

band. Src tyrosine phosphorylation in early passage HUVECs was induced sixfold by FGF-1 as compared to a 1.4-fold induction in senescent cells, whereas a threefold induction of the tyrosine phosphorylation of cortactin was observed in early passage HUVEC treated with FGF-1 as compared to no induction in senescent cells. A second experiment showed similar results (data not shown). These data suggest that while senescent HUVEC possess the ability to use the FGFR-1 pathway to regulate the FGF-1-induced mRNA levels of various cell cycle-specific genes, they are impaired in their ability to signal the tyrosine phosphorylation of the FGFR-1 substrates Src and cortactin.

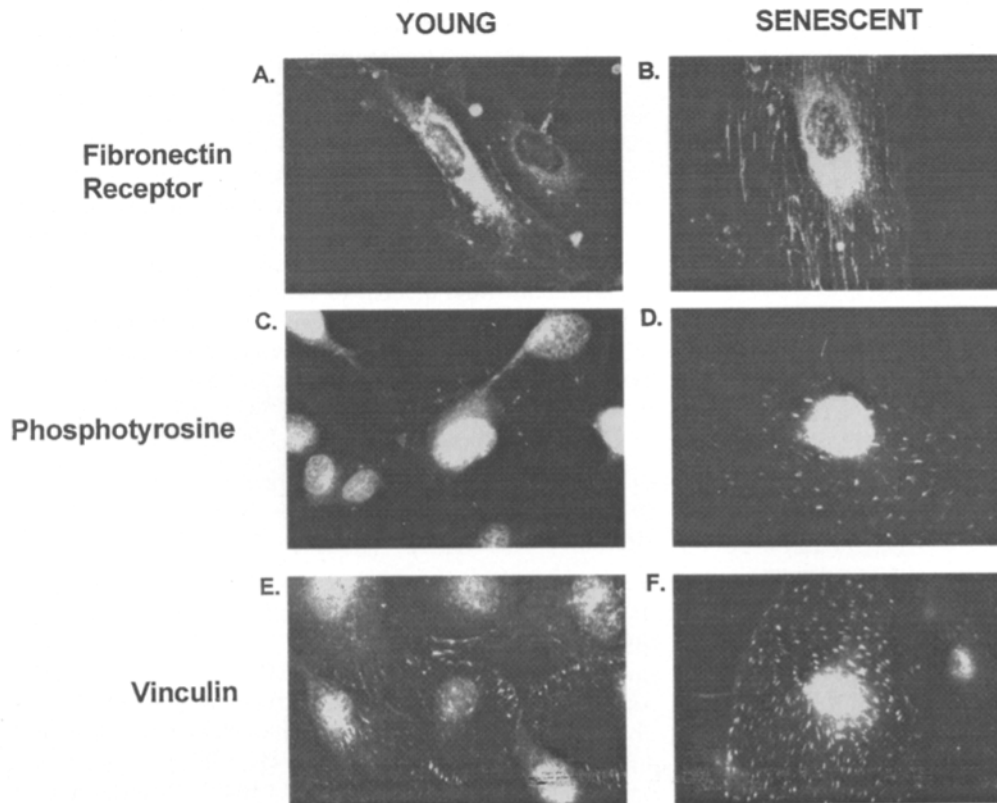
Because it is likely that the FGF-1-signaling pathway involves the association of the FGFR-1 with Src and the association of Src with cortactin (53) and the FGF-1-dependent tyrosine phosphorylation of Src was impaired in senescent HUVEC, we analyzed the activity of Src in presenescent and senescent HUVEC populations using an in vitro kinase assay. Immunoprecipitation of Src followed by an in vitro kinase assay using the substrate enolase demonstrated that a FGF-1-dependent induction of Src kinase activity was observed in the early passage HUVEC (Fig. 3). In contrast, Src-specific immunoprecipitation from senescent HUVEC populations revealed that a low level of Src tyrosine kinase activity was independent of the presence of FGF-1. The specific Src kinase activity calculated by dividing the intensity of the enolase band by the intensity of the Src protein band revealed a baseline level of Src tyrosine kinase activity in the presenescent untreated, as well as the senescent, HUVEC populations (Fig. 3 B). We evaluated the phosphorylation of enolase as a measure of specific Src tyrosine kinase activity because the autophosphorylation of Src has been inconsistent in many experiments performed and was often too low to accurately quantitate. Despite the levels of phosphorylated Src observed in Fig. 3 A, which by densitometric scanning shows a twofold induction by FGF-1 in both cell populations, we hesitate to state that this is a specific FGF-1 effect because of the inconsistencies that we have observed. Thus, in senescent HUVEC, Src is unable to phosphorylate its substrate cortactin but shows a baseline level of enzymatic activity to phosphorylate enolase, which is independent of the presence of FGF-1.

#### ***Senescent HUVEC Do Not Migrate in Response to FGF-1, Which Correlates with Prominent Focal Adhesion Sites***

It has been determined that inhibition of migration of human fibroblasts and bovine aortic endothelial cells occurs during in vitro senescence (1, 24, 29, 34). Because we have established that senescent HUVEC are defective in their ability to induce the phosphotyrosine of FGFR-1 substrates and an increase in tyrosine phosphorylation has been observed in migrating HUVEC (38), we questioned whether senescent HUVEC were capable of migration in response to FGF-1. In addition, because our previous results demonstrated that senescent HUVEC exhibited elevated levels of IL-1 $\alpha$  (15), we also analyzed the ability of early passage HUVEC to respond to IL-1 $\alpha$  as a chemoattractant. While FGF-1 was able to elicit a migratory response in presenescent HUVEC populations, neither IL-1 $\alpha$



**B**



**Figure 4.** Senescent HUVEC do not migrate in response to FGF-1 and exhibit an abundance of focal adhesion sites. (A) Presenescent (Young) and senescent HUVEC monolayers from strain H3605 were used for migration assays as described in Material and Methods. The number of cells migrating from the wound origin were counted with a light microscope at 100 magnification using a grid, and the values represent the mean of five fields with each condition tested in duplicate and three separate experiments performed. (B) Presenescent (Young) and senescent HUVEC were processed for immunofluorescence as described in Materials and Methods. The cells were photographed with a fluorescence microscope at 600 magnification (Olympus Corp.). Presenescent (Young; A, C, E) and senescent (B, D, F) HUVEC were stained with antibodies for the human fibronectin receptor ( $\alpha 5\beta 1$ ; A and B), phosphotyrosine (C and D), and vinculin (E and F).

nor PMA alone had this effect (Fig. 4 A). In addition, the effect of FGF-1 on presenescent populations was inhibited in the presence of IL-1 $\alpha$ , and these effects were specifically reversed by IRAP (9), an antagonist of IL-1 $\alpha$  action,

whereas PMA inhibited the FGF-1-induced migration regardless of the presence of IRAP. Moreover, FGF-1 was unable to induce the migration of senescent HUVEC.

In general, a decrease in cell motility correlates with

prominent local adhesions (19). Thus, the inability of senescent HUVEC to migrate in response to FGF-1 prompted us to investigate the distribution of focal adhesion sites (FAS) in presenescent and senescent HUVEC populations. Because HUVEC in culture are strongly dependent on fibronectin for their adherence (26) and the fibronectin receptor ( $\alpha 5\beta 1$ ) has been implicated in mediating cell migration (19), we used immunofluorescence techniques to analyze its distribution. As compared to presenescent HUVEC populations, senescent HUVEC demonstrated an altered distribution of fibronectin receptor-positive FAS (Fig. 4 B). The prominent FAS in senescent HUVEC were also demonstrated using antibodies against either vinculin, a protein associated with FAS, or phosphotyrosine. Thus, an altered organization of the expression of FAS in senescent HUVEC is consistent with the inability of these cells to elicit a migratory response.

## Discussion

The HUVEC is absolutely dependent upon the presence of extracellular FGF for in vitro proliferation and serial propagation (26); however, senescent HUVEC are refractory to the mitogenic potential of FGF (46). Because the nonproliferative senescent phenotype may result from defective signaling of the FGFR and aspects of the FGFR-signaling pathway have been defined in established murine cell lines (12), we analyzed normal diploid HUVEC populations for their ability to elicit a response to FGF-1. Although four FGFR family members exist, FGFR-1 is the primary transcript expressed in HUVEC. Because the level of the FGFR-1 on the surface of HUVEC is extremely low and not detectable by available methods, we examined the ability of FGF-1 to induce the expression of cell cycle-specific genes (32, 42) and the tyrosine phosphorylation of well-characterized FGFR-1 substrates (51–53).

The expression of the IME and MTL genes, Fos, ODC, and H3, was induced in both early and late passage cells treated with FGF-1. Our results are consistent with one report demonstrating the serum inducibility of cell cycle-specific genes in senescent human diploid fibroblasts (37). However, they contradict other reports that reveal the lack of serum inducibility in senescent fibroblasts (32, 42), suggesting that human endothelial cells and fibroblasts exhibit different cellular responses to growth factors and cytokines. H3 expression is usually tightly coupled to DNA synthesis, but it has been suggested that for cells to traverse the  $G_1$  phase of the cell cycle, a series of events is required and multiple pathways may act simultaneously (37). Therefore, it may be possible to induce the expression of  $G_1$  or S phase-specific genes in senescent cells, but additional signals are required for DNA synthesis and cell proliferation. Indeed, we have previously demonstrated that maximal DNA synthesis of Balb/c 3T3 cells required the presence of FGF-1 throughout the entire  $G_1$  period and withdrawal of FGF-1, or the addition of a tyrosine kinase inhibitor in late  $G_1$ , resulted in a significant reduction of DNA synthesis (51). In that regard, we have studied the tyrosine phosphorylation of FGFR-1 substrates in presenescent and senescent HUVEC. Our data suggest that unlike presenescent populations, senescent cells are impaired in their ability to induce the FGF-1-dependent tyrosine

phosphorylation of Src or cortactin and the tyrosine kinase activity of Src is not induced by FGF-1. Furthermore, we have shown that FGF-1 does not stimulate the migration of senescent HUVEC, which correlates with prominent FAS as determined by the distribution of the fibronectin receptor, vinculin and phosphotyrosine. Thus, these data imply that the induction of either IME or MTL genes by FGF-1 is not sufficient to promote a proliferative or migratory phenotype in senescent HUVEC populations. Furthermore, the lack of a FGF-1-dependent tyrosine phosphorylation of Src and cortactin implies that this part of the FGFR signal transduction pathway is impaired in senescent cells and may be involved in their inability to proliferate and/or migrate. At present, we do not know the mechanisms involved; however, it is possible that different FGFR-1 signal transduction pathways exist, one pathway resulting in the induction of gene expression through the FGFR-1 and another involving intracellular substrates coupled to the tyrosine kinase activity of the receptor. Indeed, an analysis of fusion proteins of FGF-1 and diphtheria toxin has suggested that signaling occurs by two pathways, partly through cell surface receptors and partly by transport of the growth factor into the cell cytosol and/or nucleus (48). Furthermore, a dissociation of FGF-1-stimulated mitogenesis from its tyrosine kinase activation has also been demonstrated (25). Our attempts to define further the molecular mechanisms involved in the defect in senescent HUVEC have been hampered by the inability to obtain stably transfected HUVEC populations.

Presenescent HUVEC populations exhibited a high baseline level of phosphorylated cortactin and revealed that Src was only minimally capable of inducing the phosphorylation of cortactin in response to FGF-1 (Fig. 2 B). We have considered several possibilities to explain these data. First, the difficulty in obtaining quiescent early passage HUVEC (16) may give rise to background levels of ODC and H3 expression in untreated cell populations (Fig. 1 B) and baseline levels of Src kinase activity (Fig. 3 B). Thus, the level of cortactin phosphorylation in these cell populations may reflect a residual FGF-1 effect since it is difficult to completely remove endogenous FGF-1 from this FGF-1-dependent cell. Second, RT-PCR analysis has demonstrated that the transcripts for the Src family members Fyn and Yes are expressed in HUVEC (data not shown). Thus, it may be possible that cortactin is a substrate for another Src family member that maintains cortactin in a phosphorylated state in quiescent, presenescent HUVEC. Third, it is known that cortactin has multiple potential tyrosine phosphorylation sites (50), similar to the phosphorylation of Src at different sites that is associated with its active or inactive form (5). The cortactin sites may be differentially phosphorylated to impart different activities, and thus it is possible that the overall level of phosphorylation changes little in presenescent HUVEC populations treated with FGF-1, but the site of phosphorylation is altered. The observation that only 10–30% of the total cortactin becomes tyrosine phosphorylated in Src-transformed avian and rodent cells (50) is consistent with this premise. Fourth, cortactin has been shown to bind F-actin, and this binding appears to be independent of the phosphotyrosine state of the protein (49). Thus, the FGF-1-activated Src in presenescent HUVEC may alter cortactin in



ways that are not visualized by the phosphotyrosine state of the protein. We consider this final possibility less likely since other studies have shown that increases in tyrosine phosphorylation correlate with cytoskeletal rearrangements and the localization of cortactin and F-actin correlate with the Src-dependent phosphorylation of cortactin (38, 45).

The inability of senescent HUVEC to migrate correlated with an altered organization of FAS. An inverse correlation between migration and FAS organization has also been described for fibroblasts (8). Furthermore, alterations in the expression of intracellular cytoskeletal proteins such as vinculin, which is involved with linking actin filaments to the extracellular matrix through integrins, are known to change migratory potential (11). Our results demonstrated the phosphotyrosine- and vinculin-positive FAS were localized at the periphery of early passage HUVEC. It is generally accepted that the peripheral association of FAS plays a role in cell migration (19). A migratory cell requires the ability to extend and stabilize lamellipodium where FAS are present at the cell front and rear (19). In the senescent HUVEC, prominent FAS were randomly scattered throughout the cytosol, which may act to prevent cell polarization and anchor the cells to impede their migration. In addition, because the intracellular level of IL-1 $\alpha$  has been shown to be elevated in certain strains of senescent HUVEC (15), and exogenous IL-1 $\alpha$  inhibits FGF-1-induced migration of early passage HUVEC but does not attenuate the FGF-1 induction of Src tyrosine phosphorylation (data not shown), we suggest that the consequences of elevated intracellular IL-1 $\alpha$  in senescent HUVEC may not be related to the activation of FGFR-1 substrates as they pertain to proliferation, but they may be related to the diminished migratory potential of senescent cells. Interestingly, the cytosol of senescent cell FAS seems to be the major area of accumulation of tyrosine phosphorylated proteins. Although the precise role of phosphorylated tyrosine residues in cytoskeletal proteins is not fully understood, increases in phosphotyrosine levels have been correlated with changes in the subcellular localization of Src and its substrates, and this may alter the structure and biochemical properties of focal adhesion sites, thereby affecting cell adhesion (38). However, it has also been shown that despite the importance of Y527 phosphorylation in regulating the localization of Src to focal adhesion sites in established cell lines, the amino terminus alone may be sufficient to position Src in focal adhesions (21) and Src can effect cellular adhesion in a kinase-independent manner (3). Thus, the inability of the FGFR-1 to signal Src tyrosine kinase activity in senescent HUVEC may be related to their nonproliferative and/or nonmigratory phenotype. While it is not known whether similar pathways are involved in HUVEC proliferation and migration, studies on growth factor signaling in human diploid fibroblasts through the epidermal growth factor receptor pathway have demonstrated distinct mitogenic and migratory pathways (19). Alternatively, since presenescent HUVEC populations may use exogenous FGF-1 both as a mitogen and as a survival factor, it is also possible that the withdrawal of FGF-1 from the HUVEC population may initiate an apoptotic program which may be different from the human fibroblast population.

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