

Invasion of Normal Human Fibroblasts Induced by v-Fos Is Independent of Proliferation, Immortalization, and the Tumor Suppressors p16^{INK4a} and p53

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Invasion is generally perceived to be a late event during the progression of human cancer, but to date there are no consistent reports of alterations specifically associated with malignant conversion. We provide evidence that the v-Fos oncogene induces changes in gene expression that render noninvasive normal human diploid fibroblasts highly invasive, without inducing changes in growth factor requirements or anchorage dependence for proliferation. Furthermore, v-Fos-stimulated invasion is independent of the pRb/p16^{INK4a} and p53 tumor suppressor pathways and telomerase. We have performed microarray analysis using Affymetrix GeneChips, and the gene expression profile of v-Fos transformed cells supports its role in the regulation of invasion, independent from proliferation. We also demonstrate that invasion, but not proliferation, is dependent on the activity of the up-regulated epidermal growth factor receptor. Taken together, these results indicate that AP-1-directed invasion could precede deregulated proliferation during tumorigenesis and that sustained activation of AP-1 could be the epigenetic event required for conversion of a benign tumor into a malignant one, thereby explaining why many malignant human tumors present without an obvious premalignant hyperproliferative dysplastic lesion.

The two defining hallmarks of cancer are uncontrolled proliferation and invasion (32). Invasion and subsequent metastasis create difficult problems for the clinical management and treatment of cancer. By the time of diagnosis, many cancers have invaded locally and already metastasized to distant sites (95). It is important to target the invasive component of cancer through identification of the genes that control and mediate invasion and enable metastasis.

Invasion, the migration of cells through tissue boundaries, is, under normal circumstances, a tightly controlled process that is activated in many cell types in response to extracellular signals and ceases once the stimuli are withdrawn. Invasion is complex; the invasive cell must alter its cell-cell and cell-extracellular matrix (ECM) adhesions, control the degradation of the ECM, respond to chemoattractants, rearrange the actin cytoskeleton to facilitate motility, and suppress anoikis (95, 116). The complexity of invasion and tight regulation by extracellular signals, such as growth factors, suggests that it is a transcriptionally regulated multigenic program (13, 46, 71).

Growth factors that bind to receptor tyrosine kinases stimulate proliferation and invasion (13, 34, 53, 54, 61, 113). In human tumors, the growth factor-Ras-Raf-Mek-Erk signal transduction pathway is frequently constitutively activated by mutations in component genes (20). This leads to sustained changes in the activity of transcription factors, which translate the signals into long-term phenotypic responses through changes in gene expression (14, 62, 71, 103). The activator

protein 1 (AP-1) family of transcription factors is regulated by the growth factor signal transduction pathway (26) and is important for invasion as well as proliferation (71, 76). Since AP-1 is a downstream effector of the growth factor signal transduction pathway, the sustained stimulation of its activity and subsequent changes in gene expression may be an epigenetic consequence of genetic activation of the Ras pathway by mutations in component proteins (5).

AP-1 denotes a collection of transcription factors composed primarily of heterodimers of Fos and Jun family proteins that recognize a consensus DNA sequence usually in the promoter region of target genes (43). The prototypes of each gene family were originally identified as retroviral oncogenes, thereby highlighting the importance of AP-1 to cellular transformation (18, 60, 106). Transformation by high concentrations of growth factors or oncogenes that function in the growth factor signal transduction pathway is dependent on the sustained increase in expression of AP-1 component proteins and AP-1 activity (71).

Based on the identification of an AP-1 DNA binding site in the collagenase promoter and its role in the degradation of ECM, AP-1 has been implicated in the targeting of genes involved in invasion (2, 17, 114). The epidermal growth factor (EGF)-dependent invasion of human squamous cell carcinoma-derived cell lines is dependent on AP-1 activity (61), as is the conversion of oncogenic Ras-induced hyperproliferative skin lesions into invasive carcinomas (56, 79, 80, 119). Increased expression of AP-1 component proteins, such as Fra-1, rather than mutational activation of AP-1 component proteins, is by far the most frequent means by which AP-1 activity is changed in tumors, and Fra-1 expression is associated with the acquisition of invasive potential by tumors (51, 109, 120).

Fos oncogenes can be used to specifically focus on the con-

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tribution of AP-1 to transformation, downstream of the growth factor signal transduction pathway. Transformation of immortal rat 208F fibroblasts with v-Fos^{FBR} renders the cells invasive, anchorage independent, and tumorigenic, but they remain dependent on serum for proliferation (34, 64). 208F cells can also be rendered invasive and anchorage independent by growth factor stimulation that induces the expression of AP-1 component proteins and increased AP-1 activity (34, 71). In both cases, invasion is dependent on AP-1 activity (46, 53, 54).

The ability of *Fos* oncogenes to transform cells is dependent on their functioning as transcription factors (24, 88, 115), which indicates that they mediate transformation through changes in gene expression (34, 46). Interestingly, the changes in expression of AP-1 component proteins following Fos or Ras transformation are similar, emphasizing the importance of AP-1 activation in transformation by oncogenes that function upstream of AP-1 (71). Analysis of genes differentially expressed in v-Fos-transformed 208F fibroblasts indicates that it results in the increased expression of genes that effect invasion (46, 53, 54, 71, 94). However, since v-Fos induces immortality and tumorigenicity in addition to invasion in rodent fibroblasts, it is difficult to determine if differentially expressed genes are specific effectors of invasion or play a wider role in the transformed phenotype (41). Furthermore, others have been unable to find a consistent phenotypic change associated with v-Fos expression in mortal human fibroblasts (1, 50). There is a potential role for tumor suppressors to suppress invasion, since loss or inactivation of tumor suppressors precedes invasion in most tumor model systems.

Herein we provide evidence, based on gene expression profiles, that v-Fos activates a multigenic invasion program in normal human fibroblasts in the presence of active tumor suppressor pathways (p16^{INK4a} and p53) without affecting cell cycle regulation or anchorage-dependent proliferation and suggest that this system can be used to identify genes specifically associated with AP-1 mediated invasion.

MATERIALS AND METHODS

Construction of Myc-tagged v-Fos^{FBR}. Myc-tagged v-Fos^{FBR} was generated by PCR, using pBR322 FBJ/R-MSV (65) as a template. The PCR product was ligated into pPCR-Script (Stratagene), and the sequence was verified before being cloned into pLPCX (Clontech Laboratories).

Generation of cell lines. *Telomerase*, oncogenic v-Fos, human papillomavirus type 16 (HPV16) E6, and mutant p53 were serially introduced into primary human fetal fibroblasts (hff). The hff strain was established by collagenase digestion of 16-week-old fetal skin. The cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 20 mM HEPES and 20% fetal calf serum (FCS). Early-passage hff were immortalized with the catalytic subunit of telomerase (hTERT) as described previously (68), and the cells were named Tif for "telomerase-immortalized fibroblasts."

To introduce v-Fos^{FBR} into hff and Tif, the amphotropic packaging cell lines Phoenix Ampho (from G. Nolan, Stanford University) or Retropack PT67 (from Clontech Laboratories) were transfected with the retroviral constructs pLPCX (puromycin resistance) or pLPCX FBJ/R expressing Myc-tagged v-Fos^{FBR}. The viral supernatants generated were used to infect hff/11 (22 population doublings [P.D.]) and Tif (50 P.D.) using 4 µg of Polybrene per ml. Cells were selected 8 days postinfection in 0.6 µg of puromycin per ml and puromycin-resistant colonies were pooled. Polyclonal populations were used in all experiments to avoid variation due to the clonal heterogeneity of the normal hff and Tif populations. Retroviral infections were repeated independently to confirm phenotypic observations.

p53 was inactivated in late-passage Tif by retroviral infection with either mutant p53 (from K. Ryan, Beatson Institute for Cancer Research) or HPV16 E6 (from A. Phillips, University Medical College of Georgia). Phoenix Ampho cells

were transfected with pWZL p53.273H (blasticidin) or pWZL E6 (hygromycin), and the viral supernatants were used to infect Tif. Cells were selected 8 days postinfection by using 10 µg of blasticidin per ml or 100 µg of hygromycin per ml, and pools of drug-resistant colonies were collected.

A mutant form of Fos (aFos) (from R. Hennigan, University of Cincinnati College of Medicine) was expressed in Tif-puro and Tif-Fos by nucleofection. Cells (5×10^5) were resuspended in 100 µl of NHDF-Neo (Amaxa Biosystems) with 3 µg of pEGFP-aFos and nucleofected with program T-20. The cells were incubated for 1 day before being harvested for in vitro invasion assays.

Retrovirally infected cells were screened for virus production by using a C-type reverse transcription assay from Cavid Tech (Uppsala, Sweden). All cells were routinely tested for mycoplasma contamination by Hoechst 33258 staining (12).

RNA interference. Transient expression of small interfering RNA (siRNA) was achieved by nucleofection. Tif-Fos cells (5×10^5) were resuspended in 100 µl of NHDF-Neo with 100 nM scrambled siRNA (CGAUCGAUCGAUCGAUCGA), EGF receptor (EGFR) siRNA 1 (CUCUGGAGGAAAAGAAAGU), or EGFR siRNA 2 (CACAGUGGAGCGAAUCCU) (Dharmacon). Cells were incubated for 48 h before being harvested for Western blotting, growth curve measurement, immunofluorescence, and in vitro invasion assays.

Stable suppression of EGFR expression was achieved using pSUPER.retro (Oligoengine). The puromycin selection cassette was changed to blasticidin resistance, and 64-nucleotide oligonucleotides containing an inhibitory sequence for EGFR (TTCTATGCCTTAGCAGTC) were annealed and ligated into pSUPER. Tif-Fos cells were retrovirally infected with pSUPER or pSUPER EGFR as described previously, and pools of blasticidin-resistant colonies were obtained.

P.D. and cell counting. Cells were counted at every passage, and P.D. were calculated from the formula $P.D. = 3.32(\log_{10} \text{ cells harvested} - \log_{10} \text{ cells seeded})$. Cell numbers were determined using a Casy 1 TT cell counter (Schärfe system). For cell growth curves, only attached cells were counted, but for flow cytometric analyses, adherent cells were trypsinized and combined with floating cells from the culture medium to allow analysis of the total cell population.

Western blotting. Cells were washed with phosphate-buffered saline and lysed for 30 min on ice in buffer containing 50 mM Tris (pH 8.0), 120 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40, phosphatase inhibitors (50 mM NaF and 50 µM Na₃VO₄), protease inhibitors (10 µg of aprotinin per ml, 10 µg of leupeptin per ml, and 1 mM phenylmethylsulfonyl fluoride). The extracts were cleared by centrifugation, and the supernatants stored at -70°C. Protein was quantified by the copper sulfate-bicinchoninic acid method and separated by gel electrophoresis.

Primary antibody incubations were carried out overnight at 4°C with antibodies against the following human proteins: Myc epitope (9E10; Invitrogen), p16^{INK4a} (C-20; Santa Cruz), ERK2 (clone 33; BD Transduction Labs), phospho-p44/42 MAPK (Cell Signaling), p53 (DO-1; Santa Cruz), p21^{Cip1} (clone 70; BD Transduction Labs), EGFR (clone 13; BD Transduction Labs), and activated EGFR (clone 74; BD Transduction Labs). The blots were washed and probed with horseradish peroxidase-conjugated anti-mouse or anti-rabbit antibodies (Cell Signaling) and protein detected by enhanced chemiluminescence (Amersham Pharmacia).

Immunofluorescence. Expression of Myc-tagged v-Fos was detected by immunofluorescence as described previously (97). Cells were grown on glass coverslips for at least 1 to 2 days in DMEM-20% FCS, and v-Fos was detected with 9E10 and tetramethylrhodamine-5-isothiocyanate TRITC-conjugated anti-mouse antibody while F-actin was labeled with fluorescein isothiocyanate FITC-phalloidin.

Wound-healing assay. Cell motility was assessed using a modification of the wound-healing assay (97). A wound was made in a confluent monolayer of cells by using a 200-µl pipette tip. Three independent areas were marked for identification and photographed at various time points thereafter with a digital camera attached to a phase-contrast microscope. For quantitation of wound closure, the width of each wound at various time points was measured and expressed as a percentage of the original wound size.

In vitro invasion assay. Invasion was assessed using an in vitro assay (34) with the following modifications. Cells (10^4) were seeded onto the filter of a polycarbonate chamber containing 100 µl of complete matrigel basement membrane matrix (Becton Dickinson) (in the absence or presence of AG1478). After 3 h of attachment, the cells were washed and placed in serum-free DMEM in the absence or presence of AG1478. DMEM containing 20% FCS with or without EGF in the absence or presence of AG1478 was added above the matrigel as a chemoattractant, and the assay mixture was incubated for 3 days. Invasion was visualized by staining the cells directly with 4 µM calcein acetoxymethyl ester (calcein) (Molecular Probes Europe) in serum-free DMEM for 1 h at 37°C followed by confocal microscopic analysis using a Bio-Rad MRC 600 confocal illumination unit attached to a Nikon Diaphot inverted microscope. Quantitation was performed as described previously (34).

Soft-agar assays. Anchorage independence was assessed by growth in soft agar. A base layer of 0.5% agar (both Noble and Bacto agars were used independently) in DMEM containing 20% FCS was allowed to set in six-well plates. Cells (10^4 and 10^5) were seeded in 0.3% top agar in DMEM–20% FCS. Fresh top agar in DMEM–20% FCS was added once a week, and colonies were analyzed after 3 weeks.

Flow cytometric analysis. Analysis of the cell cycle phase distribution by propidium iodide staining for DNA content was carried out as follows. Cells were trypsinized and combined with floating cells in retained medium, fixed in ice-cold 70% ethanol, and stored at 4°C. The fixation medium was aspirated after centrifugation and the cells were then stained with 10 µg of propidium iodide per ml and 250 µg of RNase A per ml for 30 min. Cell cycle analyses were made using a Becton Dickinson FACScan. No fewer than 30,000 events were processed per sample, and the bitmap was fixed to discriminate against doublets (cell or nuclear aggregates). Cell cycle phase distributions (expressed as a percentage) were obtained by decomposition of the DNA content histograms by using Cell Quest software.

Microarray analysis with Affymetrix GeneChips. Total RNA was isolated from exponentially growing cells by using the RNeasy minikit (Qiagen). RNA (15 µg) was reverse transcribed with T7(dT)₂₄ primer (Helena BioSciences). The double-stranded cDNA was in vitro transcribed with T7 RNA polymerase and biotin-labeled ribonucleotides (Affymetrix). The biotin-labeled cRNA was fragmented at 94°C for 35 min. cRNA (15 µg) was hybridized onto Affymetrix GeneChip Human Genome U95Av2 arrays at the Cancer Research UK GeneChip service, Paterson Institute for Cancer Research, Manchester, United Kingdom.

Microarray data analysis. Using Microarray Suite v5.0, all probe sets on the arrays were scaled to a target intensity of 100 (with scaling factors for each chip within threefold of each other). We excluded genes which were flagged as absent in all samples, and data were normalized either using the Robust Multichip Average method in the Bioconductor “affy” method (6) or by correcting for differences in the mean values for each chip by using GeneSpring (Silicon Genetics). The Robust Multichip Average data were subjected to analysis of variance (ANOVA) ($P < 0.05$) and significance analysis of microarrays (SAM) (105) with the false discovery rate (FDR) set to 0. The edited Venn diagram selected lists were contained entirely in both the ANOVA and SAM lists (data not shown). Genes were annotated for gene symbol, gene name, and function by using UniGene and LocusLink (National Center for Biotechnology Information) according to the HUGO gene nomenclature committee.

Materials. Mouse EGF was obtained from Sigma. The EGFR inhibitor tyrostatin AG1478 was purchased from Calbiochem CN Biosciences.

RESULTS

Expression of v-Fos in human fibroblasts. Expression of *Ras* (*Raf* and *MEK*) oncogenes in human fibroblasts (primary or telomerase immortalized) or human mammary epithelial cells that retain functional pRb/p16^{INK4a} and p53 tumor suppressor pathways results in a permanent growth arrest that is similar to replicative senescence (7, 22, 29, 30, 57, 89, 112, 122). The present study was undertaken to determine if activation of AP-1 using the *v-Fos* oncogene that functions downstream of the *Ras* signal transduction pathway could stimulate invasion and/or anchorage independence in the presence of active tumor suppressor pathways in normal human diploid fibroblasts. However, since we expected primary cells to enter into replicative senescence or possibly enter premature oncogene-induced senescence, we also used fibroblasts immortalized with the catalytic subunit of telomerase, and these are used in most of the experiments detailed here.

Normal diploid hff and Tif were infected with retroviruses encoding puromycin resistance (*puro*) or with a Myc epitope-tagged *v-Fos*^{FBJ/R} oncogene (*Fos*). Colonies of puromycin-resistant cells were pooled, and multiple polyclonal populations of cells were analyzed for the expression of v-Fos. Western analysis, performed using the 9E10 Myc epitope-specific monoclonal antibody to detect exogenous Myc-tagged v-Fos^{FBJ/R} protein is shown for representative populations of hff and Tif

and demonstrates that only cells infected with the v-Fos virus expressed a protein of the correct molecular mass, 34 kDa for v-Fos^{FBJ/R} (65) (Fig. 1A). Immunofluorescence with the Myc antibody revealed that essentially all of the cells in the hff-Fos and Tif-Fos populations expressed the protein in the nucleus of the cell and that none of the control cells, hff-puro and Tif-puro, displayed nuclear staining with anti-Myc (Fig. 1B).

v-Fos morphologically transforms human fibroblasts. The morphology of the fibroblasts expressing v-Fos was distinct from that of the puromycin-resistant control cells. At low cell density, the hff-Fos and Tif-Fos cells were more refractile and were easily distinguished from the control cells, hff, Tif, hff-puro, and Tif-puro. At high cell density, the control cells hff-puro and Tif-puro resembled the parental hff and Tif cells, where the cells aligned along their long axes and were arranged into densely packed swirls. The hff-Fos and Tif-Fos cells did not align with each other and were disorganized (data not shown).

Morphological transformation was evident when the actin cytoskeleton was labeled with FITC-phalloidin, demonstrating that the hff-Fos and Tif-Fos cells displayed an equivalent reduction in F-actin stress fibers compared to control cells (Fig. 1B). The effect of v-Fos on the actin cytoskeleton is consistent with previous studies of Fos transformation in other fibroblasts (53).

v-Fos increases the rate of wound healing in human fibroblasts. To determine if v-Fos increases cell motility, a wound was made in confluent monolayers of Tif-puro cells (Fig. 2A) and Tif-Fos cells (Fig. 2B) and the closure of the wound was monitored over time. Tif-Fos cells migrated into the wound earlier than did the Tif-puro cells, which displayed a lag phase in wound healing. After only 4 h, Tif-Fos cells displayed 25% wound closure compared to only 3% for Tif-puro cells. By 12 h, wound closure increased to 75 and 45% for Tif-Fos and Tif-puro cells, respectively, and by 23 h, both cell types had essentially closed the wound (Fig. 2C).

Human fibroblasts expressing v-Fos are highly invasive. A three-dimensional in vitro inverse invasion assay was used to determine if hff-Fos and Tif-Fos cells were capable of invading through reconstituted basement membrane matrix (34). The assay was configured such that invasion is a measure of a cell's ability to detach from the substrate and to migrate through matrigel. Essentially none of the parental cells (hff or Tif [data not shown]) or control cells (hff-puro or Tif-puro) invade the matrigel, as expected (Fig. 3), although these cells are capable of migrating through the pores of the filter (data not shown). However, v-Fos stimulated the invasion of both hff and Tif, as evidenced by the presence of calcein-stained cells in the matrigel (Fig. 3). Tif-Fos cells are the more invasive of the two, and this difference may be accounted for by the slightly lower expression of v-Fos in hff-Fos cells (Fig. 1A) rather than immortalization by telomerase. Expression of a mutant form of Fos that cannot bind DNA (aFos) does not stimulate invasion in Tif-puro but quite clearly inhibits invasion in Tif-Fos, indicating that v-Fos-stimulated invasion functions through AP-1 activity (Fig. 3).

v-Fos does not alter growth parameters or karyotype. Human fibroblasts expressing *Ras* pathway oncogenes prematurely enter into a permanent growth arrest that resembles senescence (89). Under the culture conditions described here, the expression of oncogenic v-Fos neither induces a premature senescence-like growth arrest in hff or Tif nor delays or pre-

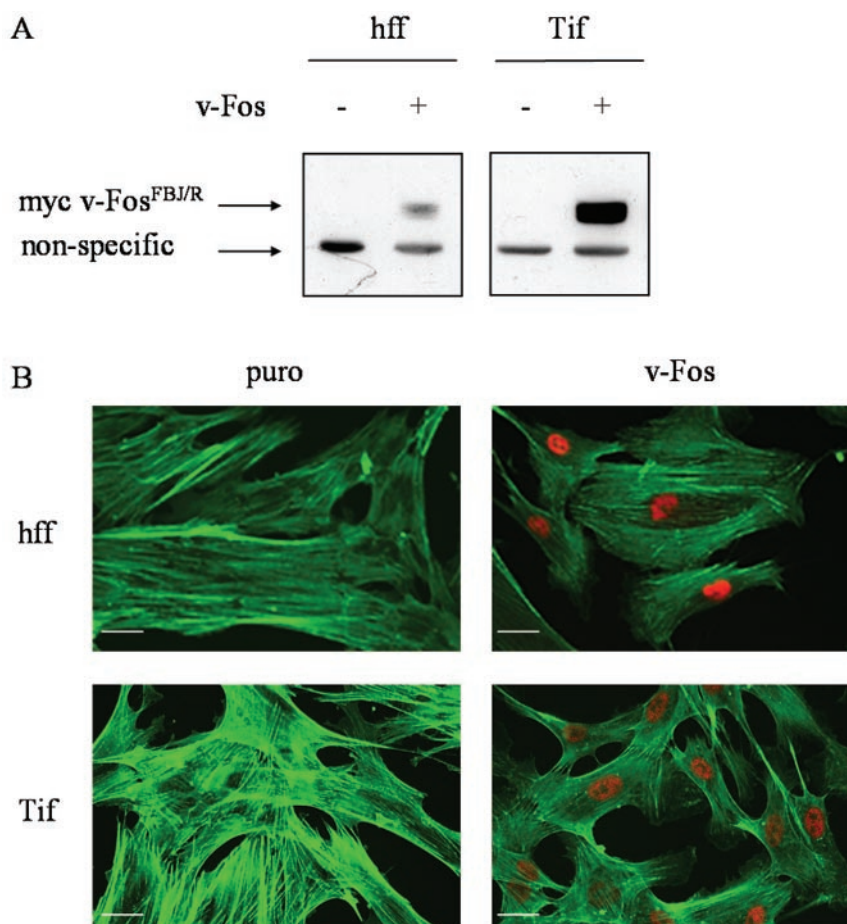


FIG. 1. Expression of v-Fos and altered morphology in human fibroblasts. Primary hff and telomerase-immortalized hff (Tif) were infected with retroviruses encoding either puromycin resistance (puro) or Myc-tagged v-Fos^{FBI/R} (v-Fos). Colonies of puromycin-resistant cells were pooled and polyclonal populations were analyzed. (A) Expression of v-Fos in the indicated cells was detected by Western blotting for Myc-tagged v-Fos^{FBI/R} with the 9E10 anti-Myc monoclonal antibody. (B) hff-puro, hff-Fos, Tif-puro, and Tif-Fos cells were costained with fluorescence-conjugated antibodies for Myc and phalloidin (TRITC and FITC respectively). Bar, 25 μ m.

vents the onset of replicative senescence in hff. The hff-puro, hff-Fos, Tif-puro, and Tif-Fos cells continue to proliferate following puromycin selection with no morphological evidence of senescence or decrease in cell doubling time. As expected, around 7 weeks after retroviral infection, both the hff-puro and hff-Fos enter into replicative senescence and express β -galactosidase, detectable at pH 6.0 (data not shown). Tif-puro and Tif-Fos cells, like parental Tif cells, are immortal and continue to proliferate indefinitely (data not shown). However, after about 80 cumulative P.D., one of the Tif-Fos populations displayed a twofold increase in saturation density, and by 100 P.D., all of the Tif-derived populations also showed a similar increase in saturation density. This was suggestive of loss of a growth-suppressing protein such as p16^{INK4a} (90). To determine if this was the case, Western analysis was performed for p16^{INK4a} with cells that had been passaged for 80 and 100 P.D. All of the cells at 80 P.D., except Tif-Fos 2, expressed p16^{INK4a}, and after 100 P.D., all of the Tif-derived cells had suppressed p16^{INK4a} expression (Fig. 4A). The loss of p16^{INK4a} was not mediated by v-Fos, nor was it a consequence of puromycin selection, since the parental Tif also suppressed p16^{INK4a} ex-

pression. The loss of expression of p16^{INK4a} was presumably due to methylation, since p16^{INK4a} expression was restored after exposure to 5-azacytidine (data not shown).

Thus, v-Fos expression neither induces nor prevents a senescence-like permanent growth arrest. In this regard, human fibroblasts are distinct from rodent fibroblasts, where v-Fos renders the cells immortal (41). The status of the tumor suppressor, p16^{INK4a}, did not affect invasion; hff-Fos cells express p16^{INK4a} and are invasive while the invasiveness of the Tif-Fos cells does not change after the loss of p16^{INK4a} with time in culture (data not shown).

Karyotyping was performed to determine if v-Fos altered chromosomal stability. Analysis of metaphase spreads revealed that hff, hff-puro, and hff-Fos cells remained diploid, 46,XY. The parental Tif cells were 46,XY with a translocation of 14q/14q, and v-Fos did not induce any additional chromosomal changes (data not shown). Hence, v-Fos does not induce gross chromosomal rearrangements.

Tif-puro and Tif-Fos cells retain a functional p53 response to DNA damage. Mutational inactivation of the p53 tumor suppressor pathway occurs in approximately 50% of human tu-

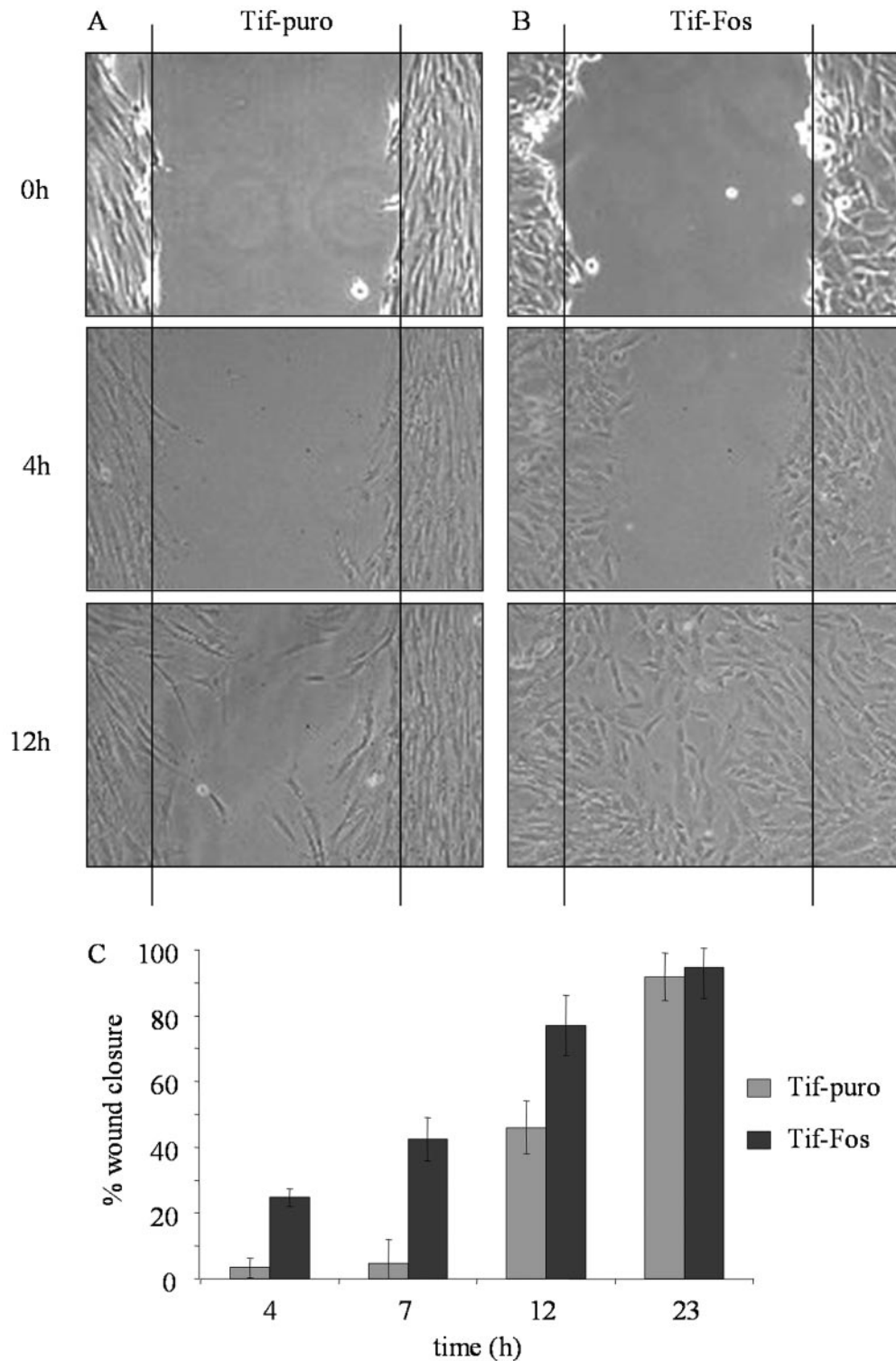


FIG. 2. Motility of telomerase-immortalized fibroblasts expressing *v-Fos*. (A and B) A wound was made in a confluent monolayer of Tif-puro (A) and Tif-Fos (B) cells, and the area was marked for identification and photographed after 0, 4 and 12 h. (C) Three independent areas of each wound made in Tif-puro (gray bars) and Tif-Fos (black bars) were measured, and the error bars show the standard deviation of the mean percent wound closure after 4, 7, and 23 h.

mors (37), and inactivation of p53 is important for immortalization and transformation of human fibroblasts by oncogenic Ras (22, 29, 30).

To determine if *v-Fos* altered p53 function, the cellular

response to DNA-damaging agents was assessed. Western blot analysis of p53 expression levels in Tif-puro and Tif-Fos cells revealed slightly elevated levels in Tif-Fos cells (Fig. 5A). However, Tif-puro and Tif-Fos cells both responded to DNA dam-

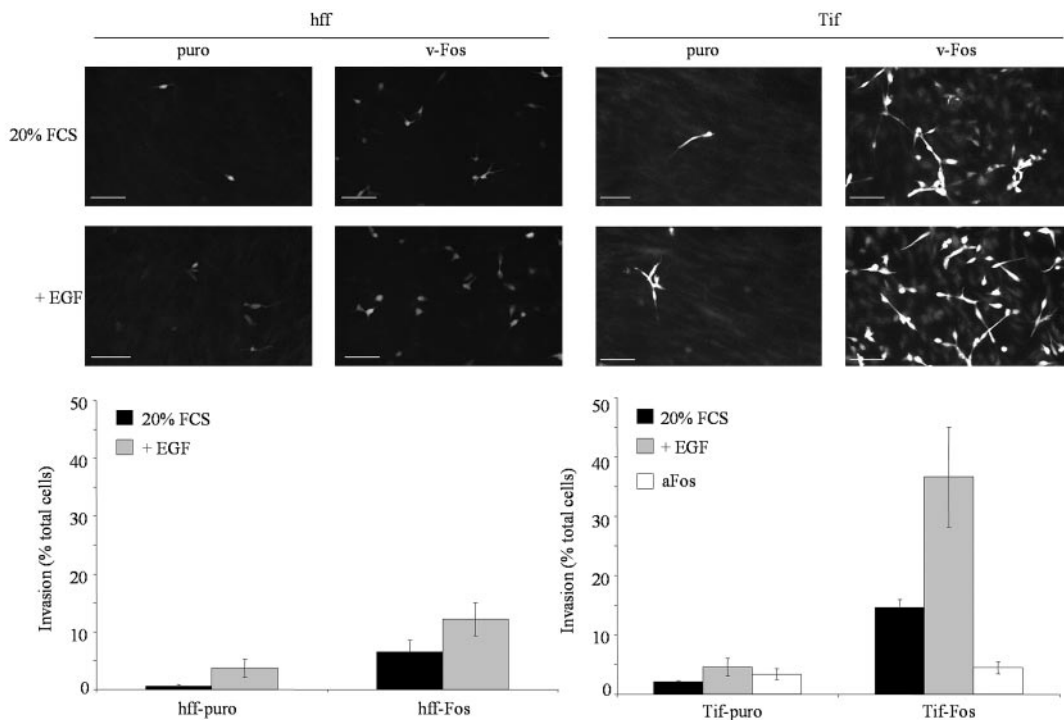


FIG. 3. Invasion of human fibroblasts expressing v-Fos. An in vitro assay was used to measure three-dimensional invasion through matrigel reconstituted basement membrane matrix. Primary hff, hff-puro and hff-Fos, and telomerase-immortalized hff, Tif-puro, and Tif-Fos were stained with calcein after 3 days and a Z-series (6 to 30 μ m) was captured on a confocal microscope and merged into one image. Bar, 100 μ m. Invasion assays were performed in duplicate, and two or three independent fields per sample were analyzed and quantitated. Invasion, in the absence (black bars) or presence of 100 ng of EGF per ml (gray bars) or aFos (white bars) was expressed as a percentage of the total number of cells seeded on the porous filter, and the error bars represent the standard deviation of the mean.

age induced by etoposide (topoisomerase II inhibitor) by stabilization of p53 protein after 2 to 8 h and a subsequent increase in p21^{Cip1/Waf1} protein expression (Fig. 5A). Flow cytometric analysis demonstrated that after 20 h, the cells emptied out of S phase and accumulated in both the G₁ and G₂ phases of the cell cycle (Fig. 5B), which is consistent with a p53-mediated response to DNA damage (8, 47). Similar results were obtained with UV-induced DNA damage, and an enzyme-linked immunosorbent assay specific for mutant p53 (Oncogene Research Products) also confirmed wild-type p53 status (data not shown). These results are consistent with previous reports that telomerase expression does not affect p53 function (42, 66, 108) and further demonstrate that v-Fos does not inactivate p53.

To demonstrate that the observed G₁ arrest was p53 dependent, p53 was inactivated in Tif, either by degradation induced by the HPV16 E6 oncoprotein (16) or by expression of a dominant negative mutant of p53, 273H (mtp53) (36). Western analysis of Tif-E6 protein extracts revealed a significant reduction in p53 expression, consistent with E6-mediated degradation of p53, while the protein extracts of Tif-mtp53 displayed high-level expression of p53, consistent with overexpression of mutant p53 (Fig. 5A). In contrast to Tif-puro and Tif-Fos cells exposed to etoposide, the majority of Tif-E6 and Tif-mtp53 cells did not display a significant increase in p21^{Cip1/Waf1} expression (Fig. 5A) and did not arrest in G₁ (Fig. 5B), consistent with the inactivation of the endogenous wild-type p53 (107, 108).

v-Fos does not alter the growth regulation of human fibroblasts. Despite inducing clear morphological transformation and rendering the cells invasive, v-Fos had no effect on growth rate, saturation density, or serum- and anchorage-dependent proliferation. The growth characteristics of the Tif-Fos cells were not radically distinct from those of Tif and Tif-puro cells. Tif-Fos grew to the same saturation density as Tif-puro when grown in 20% serum (Fig. 4B). Growth curves are shown for early-passage p16^{INK4a}-expressing cells and late-passage p16^{INK4a}-negative cells, demonstrating the twofold increase in saturation density observed in both Tif-puro and Tif-Fos cells after the loss of p16^{INK4a} expression. Tif-Fos cells, like Tif-puro cells, were serum dependent for proliferation as judged by the cessation of proliferation on being transferred from 20% serum to a low (0.2%) serum concentration (Fig. 4B).

The Tif-Fos cells also resembled Tif-puro cells in that they remain anchorage dependent for proliferation. In multiple experiments, no colonies formed in soft agar after 3 weeks (Fig. 4C). The Tif-Fos cells were tested for anchorage independence both before and after the loss of p16^{INK4a} expression, confirming that loss of the tumor suppressor p16^{INK4a} was not sufficient to render Fos-transformed human fibroblasts anchorage independent. To determine if additional loss of function of the tumor suppressor protein, p53, altered the anchorage requirements of Tif-Fos cells, p53 was inactivated by expression of either E6 or mtp53 as detailed previously. Tif-Fos-E6 and Tif-Fos-mtp53 cells remained anchorage dependent despite having inactivated both the p16^{INK4a} and p53 tumor suppress-

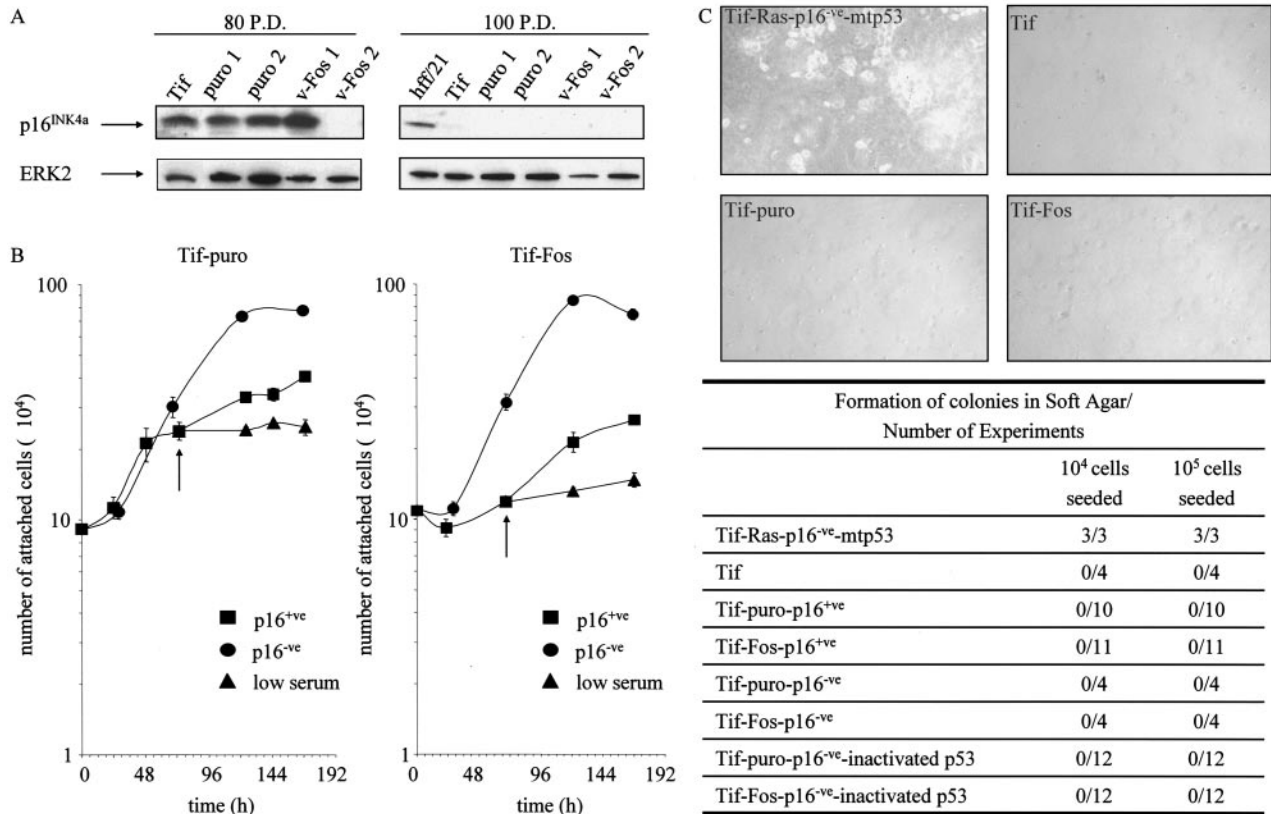


FIG. 4. *v-Fos* has no effect on proliferation or serum or anchorage dependence. (A) Telomerase-immortalized fibroblasts. Tif, Tif-puro 1, Tif-puro 2, Tif-Fos 1, and Tif-Fos 2 exhibited an increase in saturation density with each passage after 80 cumulative P.D., which correlated with a loss of p16^{INK4a} expression as demonstrated by Western blotting after 100 P.D. hff/21 (36 P.D.) was used as a positive control for p16^{INK4a}, and equal loading was determined with anti-ERK2. (B) Growth curves of telomerase-immortalized fibroblasts expressing *v-Fos*. Cells (p16^{+ve} and p16^{-ve}) were grown in 20% serum, and once exponential growth was established after 72 h, the cells were washed and incubated with either 20% serum or a low concentration of serum (0.2%), as indicated by the arrow. Cell proliferation was determined by counting the number of attached cells every day. Each experiment was performed in triplicate, and the error bars show the standard deviation of the mean daily count. (C) Anchorage-dependent growth of telomerase-immortalized fibroblasts with various combinations of *v-Fos*, loss of p16^{INK4a} (p16^{-ve}), and inactivation of p53 with HPV16 E6 or mutant p53 (273H). For each experiment, 10⁴ or 10⁵ cells were seeded in soft agar. Multiple experiments with multiple polyclonal populations of the cells were performed. During a 3-week period, colony formation was monitored and scored as 0 if there were no soft agar colonies. Tif-Ras that had lost expression of p16^{INK4a} and expressed a mutant form of p53 (mtp53) were used as a positive control for colony formation in soft agar.

sor pathways. These results indicate, as expected, that further changes are required to render human fibroblasts anchorage independent (29–31). The additional inactivation of p53 in late-passage (p16^{-ve}) Tif-puro or Tif-Fos cells does not affect the invasiveness of the cells (data not shown). Hence, invasion is independent of the two major tumor suppressor pathways, pRb/p16^{INK4a} and p53.

Gene expression profiling of invasive Tif-Fos cells. The *v-Fos* oncogene functions as a component of the AP-1 transcription factor and must mediate its phenotypic consequences, such as morphological transformation, increased motility, and invasiveness, through changes in gene expression. Since the main, if not sole, consequence of *v-Fos* expression in normal human fibroblasts is to render the cells invasive, analysis of genes differentially expressed in Tif-Fos cells should identify genes that specifically mediate invasion. Microarray analysis was performed with Affymetrix GeneChips using RNA derived from hff, Tif, Tif-puro, and Tif-Fos cells. Four independent preparations of RNA from hff and Tif were used to ensure that the differences in gene expression were not due to telomerase

expression. To exclude changes in gene expression due to puromycin selection, three independent preparations of RNA were made from two separate populations of Tif-puro. To identify changes in gene expression dependent on *v-Fos* expression, three independent RNA preparations were made from two separate Tif-Fos populations and a single RNA preparation was made from a third Tif-Fos population.

Hierarchical cluster analysis demonstrates that the global expression profiles from hff, Tif, and Tif-puro cells, although marginally distinct from each other, were closely related, while the Tif-Fos populations clustered separately (Fig. 6A). This validates the use of telomerase-immortalized fibroblasts as surrogates for primary fibroblasts for studying *v-Fos* transformation. It also demonstrates that the changes in gene expression detected in Tif-Fos cells are a consequence of *v-Fos*.

We have identified an extremely conservative list of 357 differentially expressed genes with at least a twofold change in gene expression between Tif-puro and Tif-Fos cells (Fig. 6B; Tables 1 and 2). To generate these lists, three replicate RNA samples for a single pool of Tif-puro were compared to three

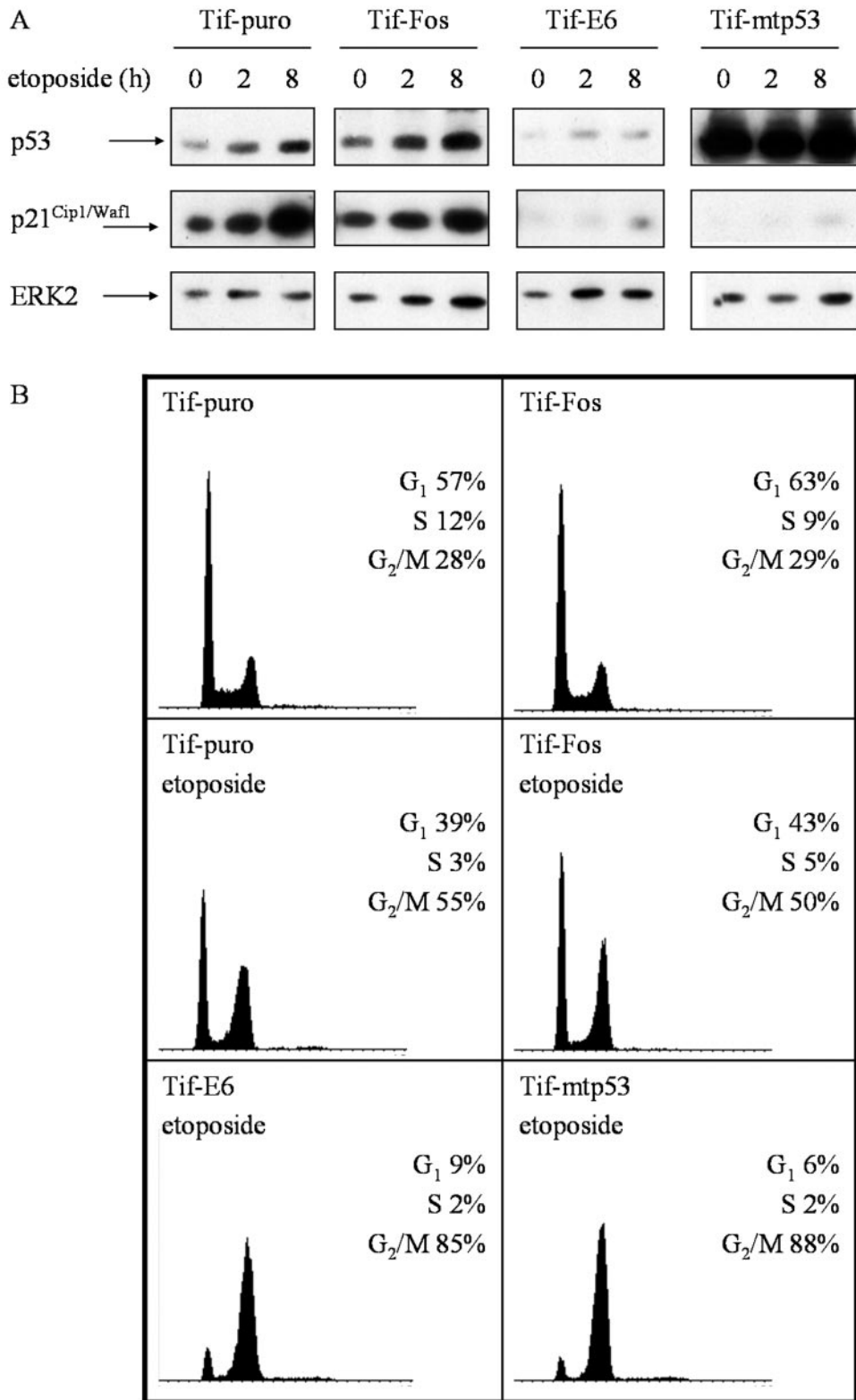


FIG. 5. v-Fos does not alter the p53 response to DNA damage. (A) Comparison of the expression levels of p53 and p21 in telomerase-immortalized fibroblasts after DNA damage induced by etoposide. Cells were treated with 1 μ M etoposide and lysed at the indicated times for Western blot analysis of p53 and p21^{Cip1/Waf1}. p53 was inactivated in Tif cells with HPV16 E6 or mutant p53 (mtp53). The membranes were stripped and reprobed for ERK2 as a loading control. (B) Flow cytometric analysis of the cell cycle phase distribution after DNA damage induced by etoposide in telomerase-immortalized fibroblasts. Exponentially growing cells were incubated in the absence or presence of 1 μ M etoposide for 20 h, stained with propidium iodide for DNA content, and analyzed by flow cytometry. The DNA content histograms are shown along with the distribution of cells in the G₁, S and G₂/M phases of the cell cycle.

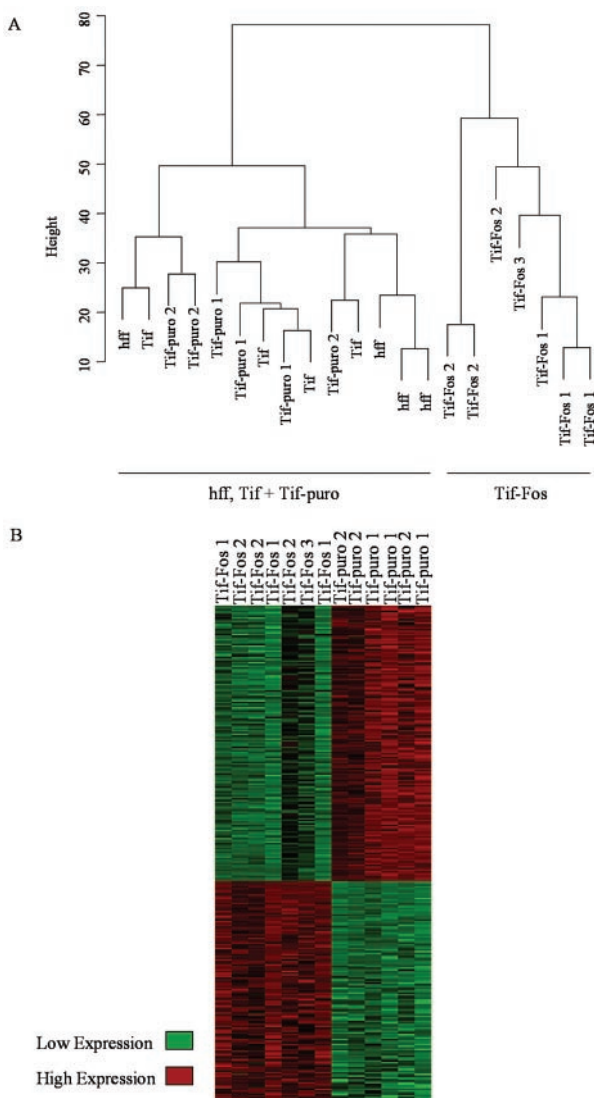


FIG. 6. Gene expression profiling. (A) Cluster dendrogram of all samples: hff ($\times 4$), Tif ($\times 4$), Tif-puro ($\times 6$), and Tif-Fos ($\times 7$). The metric used was Euclidean distance, and clustering was done by the “complete” method as implemented in the “mva” library in the “R” statistical software. (B) Statistically significant changes in gene expression assessed by SAM for all Tif-puro samples ($\times 6$) and all Tif-Fos samples ($\times 7$).

replicate RNA samples for a single pool of Tif-Fos, the genes were filtered for a fold change of twofold, and Venn diagrams were used to select genes that were common to all three lists. To ensure that the lists represented consistent changes in gene expression, each gene was checked for a twofold expression difference in all of the biological replicates not used in the initial pairwise comparison. When a gene was represented by more than one oligonucleotide on the GeneChip, the multiple representation was deleted, resulting in 130 and 227 individually represented genes up- and down-regulated respectively. Similar lists were obtained by ANOVA and SAM (Fig. 6B).

In addition to demonstrating that expression of v-Fos in human fibroblasts results in a consistent alteration of the gene expression profile, it identifies known effectors of the invasive

phenotype such as genes involved in actin cytoskeleton regulation, cell adhesion and motility, ECM and proteolysis, as well as genes that have not previously been associated with invasion. We propose that up-regulated genes function as effectors of invasion (Table 1) while down-regulated genes function as suppressors of invasion (Table 2). The paucity of cell cycle-associated genes, which supports the data presented here and previous findings that v-Fos does not alter the growth regulation of fibroblasts, is interesting (34, 64). There are a considerable number of genes differentially regulated in Tif-Fos cells that are thought to be involved in oncogenesis despite the cells remaining anchorage and serum dependent for proliferation; these include down-regulated tumor suppressors, membrane receptors, and genes involved in signal transduction, which are discussed in more detail below.

The EGFR is up-regulated by v-Fos in human fibroblasts.

The microarray analysis identified a significant increase in expression of EGFR. EGFR was up-regulated approximately 10-fold at the RNA level in all of the Tif-Fos populations. There was no difference in EGFR expression between the hff, Tif, or Tif-puro cells, indicating that the increase in expression is a consequence of v-Fos expression. Increased EGFR expression was confirmed by Western analysis of Tif-puro and Tif-Fos cells (Fig. 7A). Blotting with an antibody specific for phosphorylated EGFR reveals that the EGFR is constitutively active in the presence of v-Fos and further enhanced by EGF (Fig. 7A).

To determine EGFR tyrosine kinase activation, the response to EGF stimulation was assessed in quiescent Tif-puro and Tif-Fos cells. An increase in the amount of phosphorylated ERK was detected 15 min after treatment with EGF (100 ng/ml) by Western blotting with p44/p42 ERK phosphospecific antibody, indicating that signaling from the EGFR is functional (Fig. 7B). Pretreatment of the Tif-puro and Tif-Fos cells for 10 min with the EGFR-specific inhibitor tyrphostin AG1478 (0.5 and 1.25 μM) inhibited EGF-mediated phosphorylation of ERK, confirming that EGFR signaling was responsible for the stimulation of ERK (Fig. 7B).

To further investigate EGFR function in Tif-Fos cells, expression of the EGFR was knocked down by RNA interference by using either transient expression of EGFR siRNA or stable suppression of endogenous RNA with pSUPER EGFR. Figure 7C demonstrates that EGFR expression was successfully inhibited with siRNA against two different target sequences for EGFR after 2 days, although expression levels were starting to increase after a further 3 days. Expression of pSUPER EGFR completely suppressed EGFR expression in two individual polyclonal populations of Tif-Fos cells (Fig. 7C).

To determine whether increased expression of EGFR in Tif-Fos cells resulted in enhanced proliferation, quiescent Tif-puro and Tif-Fos cells were treated with either 10 or 100 ng of EGF per ml. EGF is a potent mitogen for human fibroblasts (9), and after 24 and 48 h, cell counts indicated that Tif-puro and Tif-Fos cells respond mitogenically to EGF in a similar manner (data not shown). However, exponentially growing cells are not dependent on EGF signaling for proliferation, since their growth was not enhanced by treatment with EGF (100 ng/ml) or, conversely, not significantly inhibited by either AG1478 (0.5 μM) or transient expression of EGFR siRNA (Fig. 7D).

TABLE 1. Selected genes up-regulated in invasive Tif-Fos cells^a

Symbol	Gene name	GenBank accession no.	Fold change
Actin cytoskeleton			
FLNB	Filamin B, beta (ABP-278)	AF042166	2.5
GEM	GTP binding protein overexpressed in skeletal muscle	U10550	2.9
KRT18	Keratin 18	M26326	39.6
LPXN	Leupaxin	AF062075	3.3
MAPRE2	Microtubule-associated protein, RP/EB family, 2	X94232	8.6
PPL	Periplakin	AF001691	30.2
SDCBP	Syndecan binding protein (syntenin)	AF000652	2.7
TNNT1	Troponin T1, skeletal, slow	AJ011712	5.5
TUBB	Tubulin, beta polypeptide	AF035316	3.4
Cell motility			
CXCL1	Chemokine (C-X-C motif) ligand 1 (MGSA)	X54489	21.2
CXCL6	Chemokine (C-X-C motif) ligand 6 (GCP-2)	U81234	37.8
LGALS3BP	Lectin, galactoside binding, soluble, 3 binding protein	L13210	5.2
ECM			
LAMA3	Laminin, α_3	L34155	5.7
RELN	Reelin	U79716	3.8
SPOCK	Sparc/osteonectin, cwcv and kazal-like domains proteoglycan (testican)	X73608	3.2
Proteolysis			
CASP1	Caspase-1, apoptosis-related cysteine protease (IL1BC)	M87507	4.5
CTSC	Cathepsin C	X87212	6.4
MMP1	Matrix metalloproteinase 1 (interstitial collagenase)	M13509	5.8
MMP3	Matrix metalloproteinase 3 (stromelysin 1, progelatinase)	X05232	21.1
PLAU	Plasminogen activator, urokinase	X02419	6.6
PRSS3	Protease, serine, 3 (mesotrypsin)	X71345	38.7
TFPI	Tissue factor pathway inhibitor (LACI)	M59499	7.9
Membrane receptors			
ADORA2B	Adenosine A2b receptor	X68487	50.1
CD9	CD9 antigen (p24)	M38690	3.2
EPHA2	EphA2	M59371	3.6
EPHB6	EphB6	D83492	8.7
EGFR	EGFR	X00588	10.9
FZD2	Frizzled homolog 2	L37882	3.8
GPRC5B	G-protein-coupled receptor C5B	AC004131	11.7
ITGA2	Integrin, α_2	X17033	6.1
ITGA3	Integrin, α_3	M59911	3.0
ITGA6	Integrin, α_6	S66213	12.5
IL1RL1	Interleukin-1 receptor-like 1 (ST2)	D12763	4.2
LIFR	Leukemia inhibitory factor receptor	X61615	4.6
MET	Met proto-oncogene (HGFR)	J02958	3.5
PTGER4	Prostaglandin E receptor 4 (subtype EP4)	L25124	14.1
PTGFR	Prostaglandin F receptor (FP)	AF004021	10.8
RA13	Retinoic acid induced 3	AF095448	4.2
RAIG-2	GPRC5B	HG4272-HT4542	5.5
SYNGR3	Synaptogyrin 3	AJ002309	5.3
TM4SF1	Transmembrane 4 superfamily, 1	M90657	19.8
TM4SF3	Transmembrane 4 superfamily, 3	M35252	188.9
Signal transduction			
ARL7	ADP-ribosylation factor-like 7	AB016811	3.9
APBB2	Amyloid beta (A4) precursor protein binding B2 (Fe65-like)	U62325	3.7
ICK	Intestinal cell kinase	AB023153	3.7
MAP4K2	Mitogen-activated protein kinase kinase kinase kinase 2	U07349	4.8
NMU	Neuromedin U	X76029	7.0
SHC3	Neuronal Shc	D84361	12.1
NMT2	<i>N</i> -Myristoyltransferase 2	AF043325	5.5
PPT2	Palmitoyl-protein thioesterase 2	AF020543	3.1
PIK3CB	Phosphoinositide-3-kinase, catalytic, beta polypeptide	S67334	2.7
PIK3CD	Phosphoinositide 3-kinase, catalytic, delta polypeptide	Y10055	7.6
PKIA	Protein kinase (cAMP-dependent, catalytic) inhibitor alpha	S76965	5.0
PTPRF	Protein tyrosine phosphatase, receptor type, F (LAR)	Y00815	3.3
PTPRK	Protein tyrosine phosphatase, receptor type, K	L77886	3.1
ARHI	Ras homolog gene family, 1 (NOEY2)	U96750	4.7
RRAS2	Ras-like protein TC21	HG1111-HT111	4.0
RHOBTB3	Rho-related BTB domain containing 3	AB020685	2.7
STAC	Src homology three (SH3) and cysteine-rich domain	D86640	2.8
Transcription factors			
CART1	Cartilage paired-class homeoprotein 1	U31986	5.0

Continued on following page

TABLE 1—Continued

Symbol	Gene name	GenBank accession no.	Fold change
FOXF2	Forkhead box F2	U13220	3.5
FOXO1A	Forkhead box O1A (rhabdomyosarcoma)	AF032885	2.8
NR2F2	Nuclear receptor 2F2	M64497	12.4
TFAP2A	Transcription factor AP-2 alpha (activating enhancer binding protein 2 alpha)	M36711	44.3
TP53	Tumor protein p53	X02469	3.5
Cell cycle and growth			
CDC20	CDC20 cell division cycle 20 homolog	U05340	3.6
CKS2	CDC28 protein kinase regulatory subunit 2	X54942	3.3
CCNB1	Cyclin B1	M25753	4.3
CDKN3	Cyclin-dependent kinase inhibitor 3	L25876	3.8
ORC2L	Origin recognition complex, subunit 2-like	U27459	3.1
Apoptosis			
BAX	BCL2-associated X protein	L22475	5.4
CARD10	Caspase recruitment domain family, 10	AL049851	5.2
CLU	Clusterin (CLI, SP-40, TRPM2, APOJ)	M25915	9.1
PTTG1	Pituitary tumor-transforming 1 (securin)	AA203476	5.1
TNFRSF10B	Tumor necrosis factor receptor superfamily, 10b (TRAIL-R2)	AF016266	3.1
TNFRSF21	Tumor necrosis factor receptor superfamily, 21 (DR6)	AF068868	2.7

^a RNA from Tif-puro ($\times 3$) and Tif-Fos ($\times 3$) cells was hybridized to Affymetrix GeneChips (human genome U95Av2), and the gene expression profiles were analyzed using GeneSpring. Filtering on fold changes in Tif-Fos cells that are greater than those in Tif-puro cells by a factor of two identified 130 genes up-regulated by v-Fos. The table lists some of the genes that may be important for various aspects of invasion. Fold changes are given as an indication of expression differences and in some cases may be overestimated if the gene is undetectable in Tif-puro cells.

EGF treatment of established rodent fibroblast cell lines results in disruption of actin stress fibers followed by morphological transformation (70). Although hff-Fos and Tif-Fos cells have a reduction in actin stress fibers compared to hff-puro and Tif-puro cells, they still have detectable stress fibers (Fig. 1B). Exposure of Tif-Fos cells to EGF (100 ng/ml) for 24 h resulted in a further reduction in actin stress fibers and a more spindle-like cell morphology, while similar treatment of Tif-puro cells with EGF had no effect on the actin cytoskeleton or cell morphology (Fig. 7E). We demonstrate that inhibition of EGFR signaling with AG1478 (1.25 μ M) can reverse the loss of stress fibers observed in Tif-Fos cells in the absence or presence of EGF (Fig. 7E). Similar results were obtained for Tif-Fos cells by using either transient EGFR siRNA or pSUPER EGFR (Fig. 7F). This indicates that the consequence of EGF signaling to the actin cytoskeleton in Tif-Fos cells is different from that observed in Tif-puro cells. Of course, other changes in gene expression may contribute to the increased responsiveness of the Tif-Fos cells to EGF, in addition to increased expression of EGFR.

The EGF-induced reduction in the number of actin stress fibers and changes in cell morphology might be expected to enhance the invasiveness of hff-Fos and Tif-Fos cells in response to EGF. Inclusion of EGF (100 ng/ml) in the upper chamber of the *in vitro* invasion assay resulted in a twofold increase in the number of cells that invade but did not result in a significant increase in the invasiveness of the hff-puro and Tif-puro cells (Fig. 3). The loss of p16^{INK4a} expression and inactivation of p53 did not affect the cells responsiveness to EGF-stimulated invasion (data not shown).

To determine if EGFR signaling was necessary for invasion of Tif-Fos cells, AG1478 was included in the invasion assay at concentrations that inhibit EGF stimulation of ERK phosphorylation (Fig. 7B). AG1478 (0.5 and 1.25 μ M) inhibited invasion of Tif-Fos cells in a dose-dependent manner and reduced invasion to a greater extent in the presence of 100 ng

of EGF per ml (Fig. 7G). Similarly, transient suppression of EGFR expression using siRNA inhibited invasion in the absence and presence of EGF (Fig. 7H). Furthermore, stable suppression of EGFR expression using pSUPER EGFR completely abolished invasion in Tif-Fos cells (Fig. 7H). This demonstrates that EGFR signaling is required for invasion of Tif-Fos cells. However, overexpression of EGFR is not sufficient to render Tif-puro cells invasive, even in the presence of EGF (data not shown).

Taken together, these results suggest that increased expression of the EGFR in human fibroblasts expressing v-Fos is necessary to maintain the invasive phenotype and validates the EGFR as a component of the AP-1-regulated multigenic invasion program.

DISCUSSION

The initiating event in many tumor progression models results in recognizable benign hyperproliferative lesions, which, through successive steps, acquire an invasive potential that completes the progression from a benign tumor into a malignant one (32). The main finding reported here is that expression of the v-Fos oncogene in human fibroblasts specifically activates the invasive program of the cell. Expression of v-Fos in human fibroblasts (primary or telomerase immortalized) alters morphology, enhances motility, and renders the cells invasive without changing their serum or anchorage requirements for proliferation, inducing a premature senescence-like growth arrest, or, in the case of hff, delaying the onset of replicative senescence. Furthermore, invasion is independent of the p16^{INK4a} and p53 tumor suppressor pathways, since v-Fos stimulates invasion in hff and Tif in which both pathways are intact. The sequential loss of p16^{INK4a} expression and p53 function does not render Tif-puro cells invasive or enhance the invasiveness of Tif-Fos cells. The separation of invasion from proliferation by the v-Fos oncogene indicates that invasion

TABLE 2. Selected genes down-regulated in invasive Tif-Fos cells^a

Symbol	Gene name	GenBank accession no.	Fold change
Actin cytoskeleton			
ACTC	Actin, alpha, cardiac muscle	J00073	-61.3
AMPH	Amphiphysin	X81438	-22.0
CALD1	Caldesmon 1	M64110	-4.1
CNN1	Calponin 1, basic, smooth muscle	D17408	-14.9
DSP	Desmoplakin 1	AL031058	-140.9
FARP1	FERM, RhoGEF (ARHGEF) and pleckstrin domain protein 1	AI701049	-4.5
FLG	Filaggrin	M60502	-48.8
HSPB1	Heat shock 27-kDa protein 1	Z23090	-2.4
LMOD1	Leiomodin 1 (smooth muscle)	X54162	-6.1
MAP1A	Microtubule-associated protein 1A	W26631	-4.3
MAP1B	Microtubule-associated protein 1B	L06237	-3.5
MYO1D	Myosin ID	AB018270	-4.6
KIAA09921	Palladin	AB023209	-7.4
TES	Testis-derived transcript (3 LIM domains)	AL050162	-8.3
TPM1	Tropomyosin 1 (alpha)	M19267	-3.2
Cell adhesion			
FAT	FAT tumour suppressor homolog 1	X87241	-2.7
ISLR	Immunoglobulin superfamily containing leucine-rich repeat	AB003184	-5.7
IGSF4	Immunoglobulin superfamily, 4	AL080181	-19.4
MFG8	Milk fat globule-EGF factor 8 protein	U58516	-6.1
OPCML	Opioid binding protein/cell adhesion molecule-like (OBCAM)	U79251	-27.3
TRO	Trophinin	AB029037	-9.6
Cell motility			
CXCL12	Chemokine (C-X-C motif) ligand 12 (SDF1)	U19495	-14.3
CTGF	Connective tissue growth factor	X78947	-21.9
ENPP2	Ectonucleotide pyrophosphatase/phosphodiesterase 2 (autotaxin)	L35594	-24.8
EFNB2	Ephrin-B2	AI765533	-3.2
MDK	Midkine (NEGF2)	X55110	-14.6
SEMA3C	Semaphorin 3C/formerly semaphorin E	AB000220	-28.8
SEMA7A	Semaphorin 7A/formerly semaphorin L	AF030698	-5.8
ECM			
DKFZp56411922	Adlican	AL049946	-2.5
CRTL1	Cartilage-linking protein 1	U43328	-83.6
CSPG2	Chondroitin sulfate proteoglycan 2 (versican)	X15998	-204.5
COL8A1	Collagen, type VIII, alpha 1	X57527	-7.1
COL15A1	Collagen, type XV, alpha 1	L25286	-59.4
COL16A1	Collagen, type XVI, alpha 1	M92642	-5.5
EFEMP1	EGF-containing fibulin-like ECM protein 1	U03877	-9.5
ELN	Elastin (SVAS, WBS)	X52896	-39.9
FBN1	Fibrillin 1 (MFS1)	X63556	-4.2
FMOD	Fibromodulin	U05291	-5.6
FN1	Fibronectin 1	X02761	-2.9
FBLN1	Fibulin 1	U01244	-13.8
HSPG2	Heparan sulfate proteoglycan 2 (perlecan)	M85289	-4.1
HAS2	Hyaluronan synthase 2	U54804	-163.3
LAMA2	Laminin, α_2 (merosin)	Z26653	-10.1
LAMA4	Laminin, α_4	S78569	-6.0
LTBP1	Latent transforming growth factor beta binding protein 1	M34057	-48.0
LTBP2	Latent transforming growth factor beta binding protein 2	Z37976	-11.5
LUM	Lumican	U21128	-52.8
MAGP2	Microfibril-associated glycoprotein-2	U37283	-17.1
MFAP4	Microfibrillar-associated protein 4	L38486	-10.6
SGCD	Sarcoglycan, delta (35DAG)	X95191	-30.1
SPP1	Secreted phosphoprotein 1 (osteopontin, BSP1, ETA-1)	AF052124	-10.2
SULF1	Sulfatase 1	AB029000	-73.3
THBS1	Thrombospondin 1	X14787	-23.5
Proteolysis			
ADAMTS2	A disintegrin-like and metalloprotease with thrombospondin type 1 motif 2	AJ003125	-19.6
CPE	Carboxypeptidase E	X51405	-8.3
C1R	Complement component 1, r subcomponent	M14058	-5.3
C1S	Complement component 1, s subcomponent	J04080	-4.3
RECK	Reversion-inducing-cysteine-rich protein with kazal motifs	D50406	-11.5
SERPING1	Serine (or cysteine) proteinase inhibitor, clade G (C1 inhibitor) 1	X54486	-8.5
TFPI2	Tissue factor pathway inhibitor 2 (PP5)	D29992	-29.6
TIMP1	Tissue inhibitor of metalloproteinase 1 (EPA, CLG1)	D11139	-2.9
Membrane receptors			
ACVR2	Activin A receptor II	D31770	-3.3

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TABLE 2—Continued

Symbol	Gene name	GenBank accession no.	Fold change
F2RL1	Coagulation factor II (thrombin) receptor-like I (PAR2)	U67058	-9.2
EDNRA	Endothelin receptor type A	D11151	-9.3
FZD1	Frizzled homolog 1	AB017363	-4.4
FZD7	Frizzled homolog 7	AB017365	-3.4
GPR51	G protein-coupled receptor 51	AF056085	-6.8
IL1R1	Interleukin 1 receptor 1	M27492	-2.5
PLXNA2	Plexin A2	AB007932	-4.0
PLXND1	Plexin D1	AB014520	-2.9
PTGER2	Prostaglandin E receptor 2 (subtype EP2), 53kDa	U19487	-4.3
Signal transduction			
ADCY9	Adenylate cyclase 9	AF036927	-3.3
BDKRB2	Bradykinin receptor B2	M88714	-4.0
CBLB	cas-Br-M (murine) ecotropic retroviral transforming sequence b	U26710	-4.7
CD24	CD24 antigen (small cell lung carcinoma cluster 4 antigen)	L33930	-134.4
CRABP2	Cellular retinoic acid binding protein 2	M97815	-27.8
DAB2	Disabled homolog 2, mitogen-responsive phosphoprotein	U53446	-2.7
DOK1	Docking protein 1 (p62dok)	U70987	-2.8
DOK5	Docking protein 5	AL050069	-11.2
EFS	Embryonal Fyn-associated substrate	AB001466	-8.2
GS3955	GS3955 protein	D87119	-11.1
HCK	Hemopoietic cell kinase	M16592	-3.3
QK1	Homolog of mouse quaking QK1 (KH domain RNA binding protein)	AL031781	-4.7
INHBB	Inhibin, beta B (activin AB beta polypeptide)	M31682	-51.4
MRAS	Muscle Ras oncogene homolog	AF043938	-3.4
NEK7	NIMA (never in mitosis gene a)-related kinase 7	AL080111	-2.8
PFTK1	PFTAIRE protein kinase 1	AB020641	-3.9
PDE4B	Phosphodiesterase 4B, cAMP-specific (PDE4 dunce homolog)	L20971	-17.8
PTN	Pleiotrophin (HBGF8, NEGF1)	M57399	-8.4
PENK	Preproenkephalin	J00123	-33.1
PTGS2	Prostaglandin-endoperoxide synthase 2 (PGG/HS, COX2)	U04636	-97.8
PRKG1	Protein kinase, cGMP-dependent 1	Y07512	-4.5
PRKCM	Protein kinase C, mu	X75756	-3.1
PTPRN	Protein tyrosine phosphatase, receptor type, N	L18983	-3.0
RAB3B	RAB3B, member RAS oncogene family	M28214	-2.6
ARHB	Ras homolog gene family, B (RhoB)	M12174	-6.2
ARHE	Ras homolog gene family, E (RhoE)	S82240	-3.7
RAC2	Ras-related C3 botulinum toxin substrate 2 (rho family, small GTPBP Rac2)	M64595	-6.5
ROR1	Receptor tyrosine kinase-like orphan receptor 1	M97675	-2.8
P164RHOGEF	Rho-specific guanine-nucleotide exchange factor	AB002335	-5.1
TESK1	Testis-specific kinase 1	D50863	-2.3
TSPAN-2	Tetraspan 2	AI924594	-5.0
TOB1	Transducer of ERBB2, 1	D38305	-2.9
TGFBR1	Transforming growth factor, beta receptor 1 (ALK-5)	L11695	-3.4
RALA	v-Ral simian leukemia viral oncogene homolog A (Ras related)	M29893	-3.4
WASF3	WAS protein family, 3 (brush-1)	S69790	-6.1
WNT2	Wingless-type MMTV integration site 2	X07876	-14.9
WNT5A	Wingless-type MMTV integration site 5A	L20861	-7.7
Transcription factors			
BHLHB2	Basic helix-loop-helix class B, 2 (DEC1)	AB004066	-4.5
CUTL1	Cut-like 1, CCAAT displacement protein	L12579	-3.9
CKTSF1B1	Cysteine knot superfamily, 1, BMP antagonist 1 (gremlin/Drm)	AF045800	-5.5
CRIP1	Cysteine-rich protein 1 (intestinal)	AI017574	-54.7
FOXD1	Forkhead box D1	U59831	-4.2
LDB2	LIM domain binding 2	AF052389	-9.4
LITAF	Lipopolysaccharide-induced TNF factor	AF010312	-4.4
NOTCH3	Notch homolog 3	U97669	-19.8
STAT1	Signal transducer and activator of transcription 1	M97935	-5.1
TCF7	Transcription factor 7 (T-cell specific, HMG-box)	X59871	-4.7
TCF21	Transcription factor 21	AF047419	-9.6
Cell cycle and growth			
CCND2	Cyclin D2	D13639	-9.0
DTR	Diphtheria toxin receptor (HBEGF)	M60278	-10.4
GAS1	Growth arrest-specific 1	L13698	-6.1
GAS6	Growth arrest-specific 6	L13720	-6.0
IGFBP3	Insulin-like growth factor binding protein 3	M35878	-9.3
IGFBP4	Insulin-like growth factor binding protein 4	M62403	-4.5
IGFBP5	Insulin-like growth factor binding protein 5	L27560	-60.6
IGFBP7	Insulin-like growth factor binding protein 7 (MAC25)	L19182	-4.3
NRG1	Neuregulin 1	M94167	-5.2
NTF3	Neurotrophin 3	X53655	-10.0

Continued on following page

TABLE 2—Continued

Symbol	Gene name	GenBank accession no.	Fold change
SFRP1	Secreted frizzled-related protein 1	AF056087	-5.2
FGF2	Fibroblast growth factor 2 (basic)	M27968	-3.4
FGF5	Fibroblast growth factor 5	M37825	-7.0
FGF7	Fibroblast growth factor 7 (KGF)	S81661	-32.7
Apoptosis			
TNFRSF6	Tumor necrosis factor receptor superfamily, member 6	X63717	-2.6
TNFA1P3	Tumor necrosis factor, alpha-induced protein 3	M59465	-5.1

^a RNA from Tif-puro ($\times 3$) and Tif-Fos ($\times 3$) cells was hybridized to Affymetrix GeneChips (human genome U95Av2), and the gene expression profiles were analyzed using GeneSpring. Filtering on fold changes in Tif-Fos cells that are less than those in Tif-puro cells by a factor of two identified 227 genes down-regulated by v-Fos. The table lists some of the genes that may be important for various aspects of invasion. Fold changes are given as an indication of expression differences and in some cases may be overestimated if the gene is undetectable in Tif-Fos cells.

could occur early during tumor progression but may not be recognized since premalignant invasive lesions would be difficult to identify. Early invasiveness could even contribute to proliferation through the loss of growth restraints imposed by the normal architecture of the tissue, the release of growth factors from the degraded ECM, and the repression of anoikis. In this manner, the invasive cells could help to create the environment in which deregulated proliferation and anchorage independence could be selected. This might help to explain why, clinically, many cancers develop without showing evidence of a benign hyperproliferative phase (95).

Gene expression profiles. Since v-Fos specifically induces invasion following expression in normal human fibroblasts, the changes in gene expression detected in Tif-Fos must account for the invasive phenotype and define the multigenic invasion program. The comparison of gene expression profiles between Tif-Fos and Tif-puro cells revealed consistent significant changes in gene expression that distinguish Tif-Fos cells from the other cells: 130 genes up-regulated and 227 genes down-regulated. The genes that were identified as differentially expressed represent the many cellular functions associated with invasion (Tables 1 and 2). Previous analyses indicate that differentially expressed genes function as part of a program and that the complete program must be intact for the cells to invade (46, 53, 54, 94).

Invasion is dependent on the increased expression of extracellular proteases and a reduction in expression of their inhibitors (15, 40, 110, 111), many of which are AP-1 regulated (17, 114). The gene expression profile of the Tif-Fos cells identifies several extracellular proteases that are up-regulated and several protease inhibitors that are down-regulated. Inhibition of AP-1 activity, which reduces the expression of proteases and increases the expression of their inhibitors, also reduces the invasiveness of the cells (19, 82, 91, 92).

More changes in gene expression in addition to proteases are necessary for AP-1-mediated invasion. Of particular note is the collection of down-regulated genes that are known tumor (oncogene) suppressor genes. Potent inhibitors of fibroblast transformation, lumican (118), fibulin 1 (75, 86), and IGFBP5 (59, 85), are expressed in mouse embryo fibroblasts that are resistant to oncogene-mediated transformation (4). Calponin 1 (100) and DOK1/p62dok (93) are also known suppressors of oncogene-mediated transformation. DAB2 is of particular interest since it suppresses signaling from the growth factor signal transduction pathway to AP-1 (33, 104) and is down

regulated in a variety of human tumors (11, 23). Other known tumor suppressor genes, those for thrombospondin 1 (63, 78), RECK (69, 81, 99), TIMP1 (77), IGSF4 (25), gremlin/CKTSF1B1 (10), sFRP1 (49, 98), IGFBP7/MAC25 (96), fibronectin 1 (52, 73, 101), STAT1 (38), and FAT (21), and some purported tumor suppressor genes, those for brush-1/WASF3 (87), TES (102), and WNT5A (39), are all down-regulated in Tif-Fos cells. The number of tumor suppressor genes that are down-regulated in Tif-Fos cells and their breadth of activities (ECM components, transcription regulators, signal transducers, and protease inhibitors) reflect the complexity of the invasion program and the many aspects of cell biology that are engaged during invasion. In support of the role of AP-1 in tumor cell invasion, rather than proliferation, most of the down-regulated tumor suppressors are not direct inhibitors of the cell cycle.

Although it remains to be determined if the down-regulation of each gene is necessary for the invasive phenotype, it demonstrates that multiple suppressors of the invasive and transformed phenotype are expressed in normal fibroblasts. To the extent that each gene is a member of the multigenic invasion program, it seems unlikely that an inactivating mutation in any one of these genes would render the cells invasive or fully transformed. This implies that the down-regulation of most of these genes is the consequence of epigenetic mechanisms such as DNA methylation or histone deacetylation. v-Fos-mediated morphological transformation and invasion are known to be dependent on the activity of DNA methyltransferases and histone deacetylases (3, 71).

Many of the up-regulated genes are also associated with multiple aspects of invasion, such as ECM components and their receptors, proteases, regulators of the actin cytoskeleton, and transcription factors. Again, there are few genes directly associated with the cell cycle. Surprisingly, many known growth factors are down-regulated in Tif-Fos, perhaps accounting for their lack of independence from growth factors. However, several receptors for growth factors, e.g., EGFR (113), met (13), EphA2 (35), and EphB6 (28), that can also affect motility or invasion were significantly up-regulated in the Tif-Fos cells.

The increase in EGFR expression in Tif-Fos cells was unexpected since it has not been demonstrated in diploid human fibroblasts, although it is well established that it is often overexpressed in a variety of human tumors (117). The AP-2 binding site in the promoter region of EGFR may be responsible for phorbol myristate acetate-induced up-regulation of the

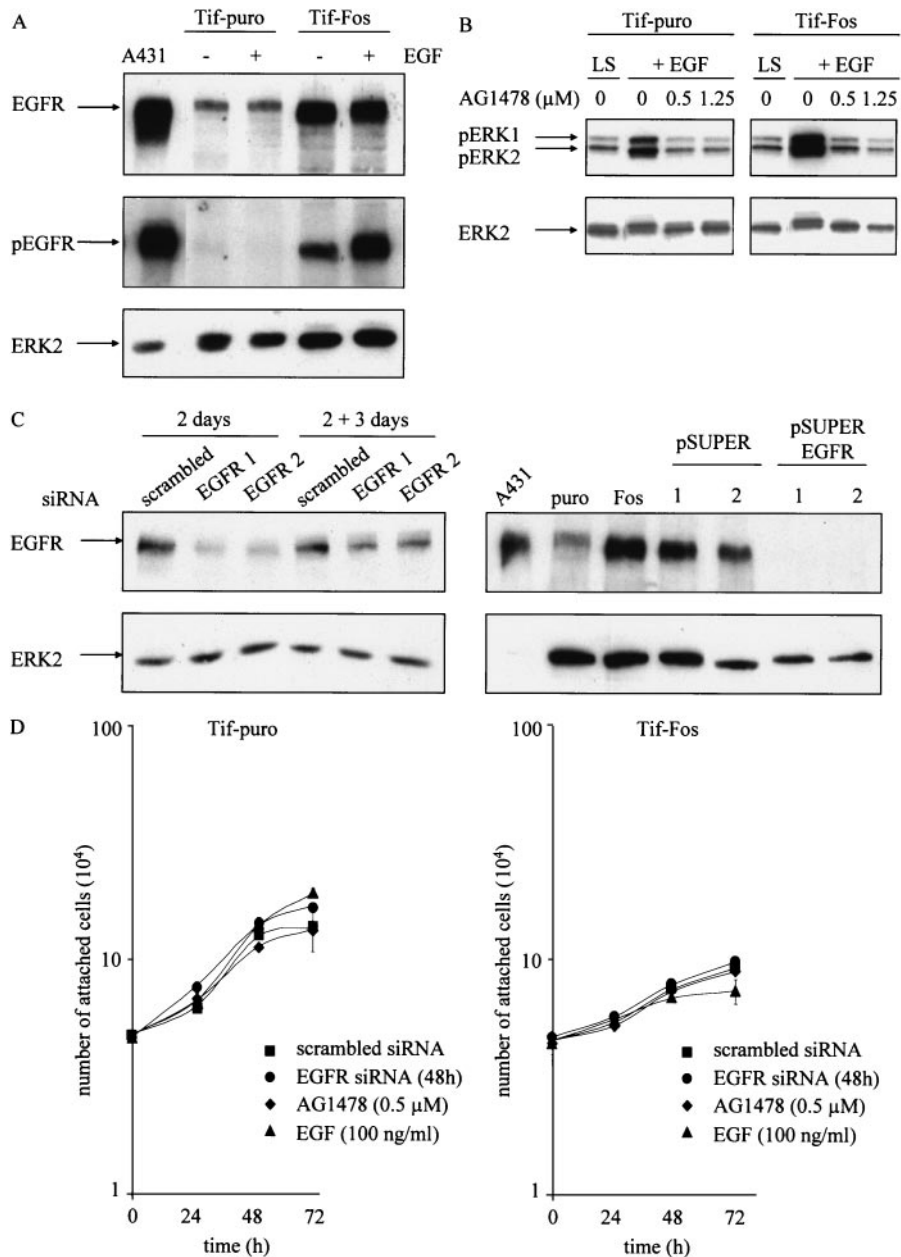


FIG. 7. Expression of EGFR and EGFR signaling in telomerase-immortalized fibroblasts expressing v-Fos. (A) Western blotting for expression of EGFR and activated EGFR (pEGFR) in Tif-puro and Tif-Fos cells (100 μ g of protein) in the absence and presence of EGF (100 ng/ml for 10 min). A431 cells (25 μ g of protein) were used as a positive control, and equal loading was determined using anti-ERK2. (B) Tif-puro and Tif-Fos cells incubated in low (0.2%) serum (LS) for 60 h were stimulated for 15 min with EGF (100 ng/ml), and phosphorylation of ERK was detected with the p44/p42 mitogen-activated protein kinase phosphospecific antibody (pERK1 and pERK2). EGFR-mediated signaling to ERK was inhibited by a 10-min pretreatment with the EGFR-specific inhibitor tyrosinase AG1478 (0.5 and 1.25 μ M). Total ERK2 is shown as a loading control. (C) Suppression of EGFR expression by RNA interference. Tif-Fos cells were nucleofected with either scrambled siRNA or EGFR siRNA (100 nM). After 2 days, the cells were lysed and EGFR expression was determined by Western blotting. Parallel cultures of cells were trypsinized and incubated in an in vitro invasion assay for 3 days, and EGFR expression was again determined by Western blotting. Tif-Fos cells were also infected with retroviruses encoding pSUPER or pSUPER EGFR. EGFR and EGFR expression was determined by Western blotting in two independent polyclonal populations of each. A431 cells were used as a positive control, and ERK2 expression was determined as a loading control. (D) Tif-puro and Tif-Fos cells were nucleofected with siRNA. After 48 h, proliferation was assessed by counting the attached cells in the absence or presence of 100 nM EGFR siRNA, 0.5 μ M AG1478, or 100 ng of EGF per ml. (E) Tif-puro and Tif-Fos cells were stained for actin stress fibers with phalloidin-FITC in the absence or presence of EGF (100 ng/ml) for 24 h with or without pretreatment (10 min) with AG1478 (1.25 μ M). Fos expression was detected with the 9E10 anti-Myc monoclonal antibody and anti-mouse-TRITC. Bar, 100 μ m. (F) Tif-Fos cells transiently expressing scrambled or EGFR siRNA (100 nM for 72h) or stably expressing pSUPER or pSUPER EGFR were stained with phalloidin-FITC in the absence or presence of EGF (100 ng/ml) for 24 h. Fos expression was detected with the 9E10 anti-Myc monoclonal antibody and anti-mouse-TRITC. Bar, 100 μ m. (G) Invasion of Tif-Fos in the absence (black bars) or presence of AG1478 (0.5 and 1.25 μ M, gray bars) with or without EGF (100 ng/ml) for 3 days. Invasion was quantitated as described in Materials and Methods and normalized with respect to invasion of Tif-Fos alone. The error bars represent the standard deviation of the mean change. (H) Invasion of Tif-Fos cells transiently expressing scrambled (black bars) or EGFR (gray bars) siRNA 2 days after nucleofection, in the absence or presence of EGF (100 ng/ml) for 3 days or stably expressing pSUPER (black bars) or pSUPER EGFR (gray bars). Invasion is normalized with respect to invasion of Tif-Fos alone, and the error bars represent the standard deviation of the mean change.

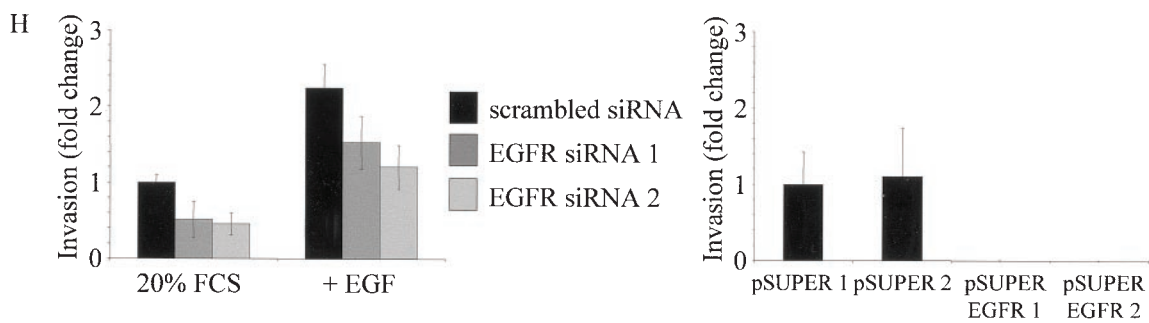
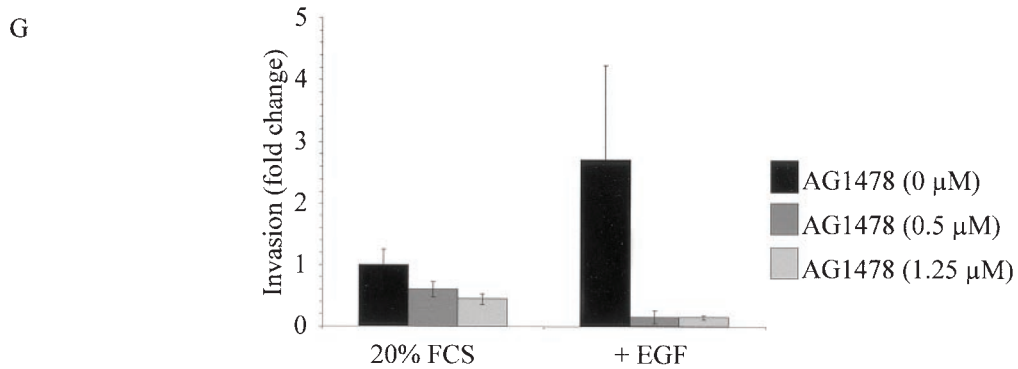
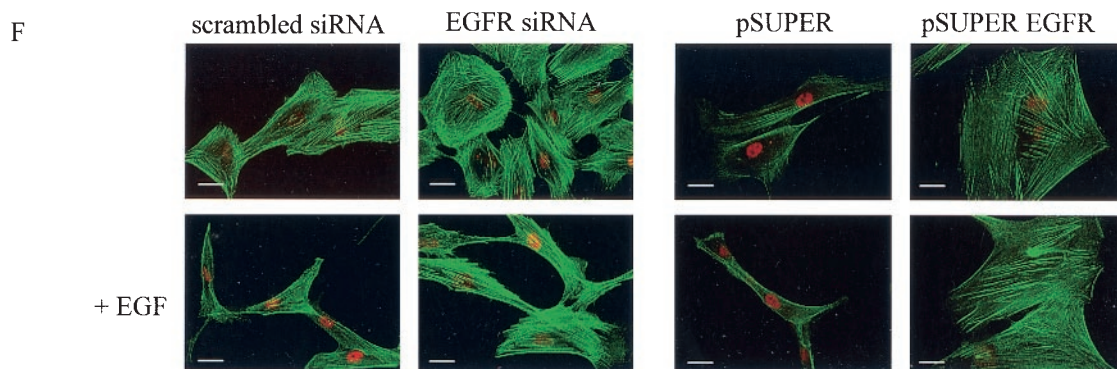
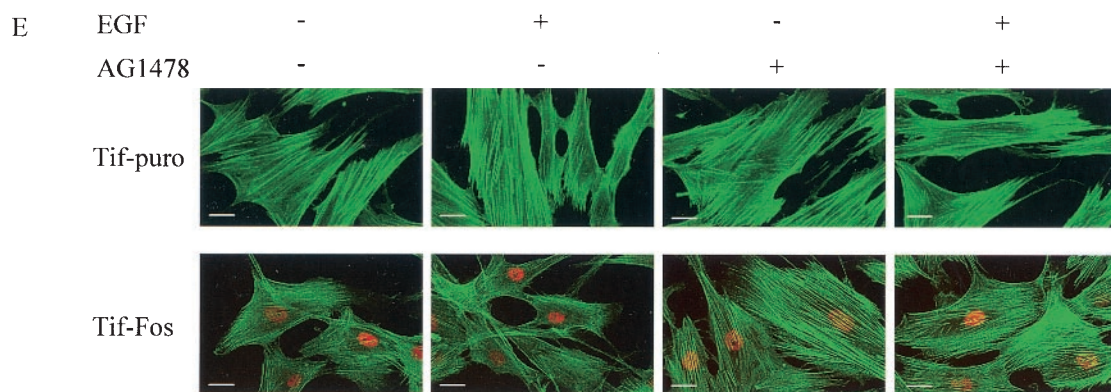


FIG. 7—Continued.

EGFR (44). The transcription factor AP-2 is notably up-regulated in Tif-Fos. AP-1 also regulates EGFR expression (45, 55, 121). In Tif-Fos, either or both of AP-2 and AP-1 could account for the up-regulation of EGFR expression.

Increased expression of the EGFR is often associated with invasiveness (113). It is known to signal to the actin cytoskeleton and to stimulate cell motility and invasion. In squamous cell carcinoma-derived cell lines that overexpress the EGFR, its activity is required for cellular invasion (61). Tif-Fos cells require EGFR activity for invasion, and inhibition of tyrosine kinase activity dramatically inhibits the invasiveness of the cells in the presence or absence of added ligand. However, EGFR activity in the presence of serum is not necessary for proliferation even though EGF is mitogenic for Tif-puro and Tif-Fos cells. This suggests that increased EGFR is signaling primarily to the invasive phenotype. Contrary to expectation, the known ligands for EGFR are not expressed in Tif-Fos cells. Thus, there is no evidence that an autocrine system for invasion, functioning through ligand binding to the EGFR, occurs in Tif-Fos cells (113). However, there are multiple ways of activating the EGFR through other signaling pathways such as lysophosphatidic acid (27), integrins (67), or prostaglandins (72).

The downstream pathways activated by the EGFR that are important for invasion remain to be elucidated. In the simplest sense, it could be serving as a guidance function and regulating cytoskeleton reorganization for motility (113), in a more complex view, it could be performing that function as well as stimulating changes in gene expression that further enhance invasiveness (53). With regard to a possible role in guidance, it has been demonstrated that in the presence of the EGFR, α_6 -integrin mediates laminin-mediated chemotaxis in fibroblasts (58, 74). In another system, EGFR activation stimulates the expression of α_2 -integrin, integrin-mediated adhesion, and motility (83). Both α_2 - and α_6 -integrin are up-regulated in Tif-Fos cells according to microarray analysis. The up-regulation of α_2 -integrin may also contribute to the invasiveness of the cells through the activation of its cytoplasmic domain by R-Ras2, which is also up-regulated in Tif-Fos cells (48). Phosphoinositide-3-kinase catalytic subunit p110delta is up-regulated in Tif-Fos cells and has been shown to regulate EGF-stimulated motility in breast cancer cells by controlling the directionality and speed of migration (84).

The changes in gene expression in human diploid fibroblasts following expression of the *v-Fos* oncogene must account for the increased invasiveness displayed by Tif-Fos cells. Further functional analysis will establish which differentially expressed genes contribute to the invasive phenotype and comprise the AP-1 regulated invasion program. The lack of changes in expression of genes that regulate cell cycle and the specific down-regulation of genes encoding polypeptide growth factors forms the basis of the activation of the invasive program in the absence of the proliferative program of the cells.

These results establish that activation of AP-1 through *v-Fos* specifically confers an invasive phenotype upon human fibroblasts that is independent of anchorage independence and cell cycle alterations normally associated with oncogenic transformation (31). The independence of invasion from these cell cycle tumor suppressor genes indicates that the invasion pro-

gram can be activated in their presence, as would be expected for a normal cellular invasion program.

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