Aging Fibroblasts Present Reduced Epidermal Growth Factor (EGF) Responsiveness Due to Preferential Loss of EGF Receptors*

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Hidenori Shirahara, Kiran Gupta, Kathryn Drabik and Alan Wells

From the Department of Pathology, University of Pittsburgh, Pittsburgh, PA 15261 and ‡Department of Pathology, University of Alabama, Birmingham, Alabama 35294-0007

Wound healing is compromised in aging adults in part due to decreased responsiveness of fibroblasts to extracellular signals. However, the cellular mechanisms underlying this phenomenon are not known. Aged dermal fibroblasts with reduced remaining replicative capacities demonstrated decreased epidermal growth factor (EGF)-induced cell migratory and proliferative capacities, as reported previously. Thus, as cells approach senescence, programmed in vivo or in vitro, EGF responsiveness is preferentially lost. To define the rate-limiting signaling event, we found that the activity of two different EGF receptor (EGFR)-signaling pathways to cell migration (phospholipase-C \( \gamma \)) and/or mitogenesis (extracellular signal-regulated-mitogen-activated kinases) were decreased in near senescent cells despite unchanged levels of effector molecules. Aged cells presented decreased levels of EGFR, although insulin receptor and transferrin receptor levels were relatively unchanged. EGFR mRNA levels and production of new transcripts decreased during aging, suggesting that this preferential loss of EGFR was due to diminished production, which more than counteracts the reduced ligand-induced receptor loss. Since these data suggested that the decrement in EGF was rate-limiting, higher levels of EGFR were established in near senescent cells by electroporation of EGFR cDNA. These cells presented higher levels of EGFR and recovered their EGF-induced migration and proliferation responsiveness. Thus, the defect in EGFR responsiveness of aged dermal fibroblasts is secondary to reduced EGFR message transcription. Our experimental model suggests that EGFR gene delivery might be an effective future therapy for compromised wound healing.

Problems in wound and skin repair constitute major medical problems for aging adults. The age-related loss of wound healing capacity leads to a high risk of surgical wound dehiscence and infection (1, 2). All phases of wound healing are diminished (3, 4). In normal wound healing, fibroblasts are recruited from the surrounding intact tissue into the granulation tissue to surround the surrounding intact tissue into the granulation tissue to proliferate and regenerate a new dermal layer in response to various factors presented in the wound fluid (5). Thus, both fibroblast mitogenicity and mitogenesis are critical for wound repair. During aging dermal fibroblasts lose both proliferative and basal migratory capacity (3); this seems to be a major reason for compromised wound healing in aged adults. To compensate, growth factors have been used as adjuvants in non-healing wounds with limited success (6–10). We propose that understanding the molecular bases underlying this age-associated decline in these capacities will allow for more rational and successful modulation of wound repair.

Fibroblast motility and proliferation are regulated by numerous growth factors (11); among those that are most robust are factors that activate the epidermal growth factor receptor (EGFR)\(^1\) (12–16). These factors, transforming growth factor-\( \alpha \) and heparin-binding EGF-like growth factor in particular (17–19), are present during all stages of wound repair, suggesting that they play important roles in orchestrating wound repair. EGFR levels on dermal fibroblasts have been seen to decline in aging, with this decline correlating with decreased mitogenic responsiveness to EGF (20, 21). The other critical cell property induced by EGFR signaling, cell motility, has not been investigated during aging. It has been reported that endothelial cell proliferative and migratory responses to a different growth factor, fibroblast growth factor, decrease as human umbilical vein endothelial cell senesce (22). However, since fibroblasts did not demonstrate a similar correlation between aging and fibroblast growth factor nonresponsiveness (23) and human umbilical vein endothelial cells are dependent on fibroblast growth factor for growth, the causal nature of this correlation between senescence and responsiveness to growth factors is uncertain and remains to be demonstrated. Furthermore, the crucial question of whether this concomitant decline in EGFR levels and cell responsiveness is causally or only coincidentally related to a global cellular decline in functioning remains unknown.

Recent advances in signal transduction research have defined intracellular signaling pathways that are required for both motility and mitogenesis. Full EGF-induced cell migration requires phospholipase-C \( \gamma \) (PLC\( \gamma \)) signaling (24, 25). Inhibition of PLC\( \gamma \) signaling specifically abrogates cell motility but not proliferation (24, 26); thus, activation of this pathway could be thought of as an indicator of EGF-mediated cell locomotion. Another major signaling pathway from EGFR is via mitogen-activated protein kinase (MAPK) signaling pathway, which is required for both cell mitogenesis and cell migration (27, 28); the point of divergence of these two cell responses

\( ^1 \)The abbreviations used are: EGF, epidermal growth factor receptor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GFP, green fluorescence protein; MAPK, mitogen-activated protein kinase; PDR, population doublings remaining; PLC\( \gamma \), phospholipase C\( \gamma \); Erk, extracellular signal-regulated kinase; DMEM, Dulbecco’s modified Eagle’s medium; FBS, fetal bovine serum; PAGE, polyacrylamide gel electrophoresis; BrdUrd, bromodeoxyuridine.

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‡ To whom correspondence should be addressed: Dept. of Pathology, University of Pittsburgh, ST13 Scaife, Terrace St., Pittsburgh, PA 15261. Tel.: 412-624-0973; Fax: 412-647-8567; E-mail: wells3@pitt.edu.

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occurs downstream of MAPK kinase in this pathway (28) but remains to be deciphered. Despite the uncertainty of all the signals required for either mitogen or mitogenesis, these two intracellular effectors, PLCγ and Erk-MAPK, provide intracellular barometers of signal transduction and intermediary markers of cell locomotive and proliferative responses, respectively.

We hypothesized that decreased EGFR expression causally results in impaired responsiveness of dermal fibroblasts. We measured negative effects of cell aging on EGF-induced cell motility. Basal and EGF-induced cell motility were measured as described under “Experimental Procedures.” The data are the mean ± S.E. of more than three independent studies performed in triplicate. Statistical analysis was performed by Student’s t test as compared with P5 (PDR42) of Hs68; * p < 0.05, **, p < 0.01 C, fibroblasts from fetus male (Δ, basal; ■, with EGF), 1-month-old male (CRL-1489) (○, basal; ●, with EGF), 17-year-old male (CRL-7815) (△, basal; ▲, with EGF), and 83-year-old male (CRL-7815) (○, basal; ●, with EGF) were assessed by cell migration assay. Fibroblasts from 1-month-old male (CRL-1489) and 83-year-old male (CRL-7815) were passaged and assessed at indicated passages. Basal and EGF-induced cell motility were measured as described under “Experimental Procedures.” The data are the mean ± S.E. of more than three independent studies performed in triplicate. Statistical analysis was performed by Student’s t test as compared with fetal cells: *, p < 0.05, **, p < 0.01.

**EXPERIMENTAL PROCEDURES**

Reagents—Hs68 and other normal human diploid fibroblasts were purchased from American Type Culture Collection (ATCC, Rockville, MD); 23-week male fetus CRL-1475 (obtained at passage 8; hereafter referred to as P8), 1-month-old male CRL-1489 (P8), 17-year-old male CRL-7315 (P5), 83-year-old male CRL-7815 (P3); 10-month-old female CRL-1497 (P6); 84-year-old female CRL-7321 (P3). Human diploid fibroblasts from 16-week female fetus (GM04522A, P6) and 19-year-old female (GM08399, P5) were purchased from the NIA cell repository (Camden, NJ). Cells were passaged by 1:6 split to increase cumulative cell population doubling level by 3 on each passage (29). Population doublings remaining (PDR) was back-calculated from passaging cells to senescence; PDR indicates the remaining replicative capacity, so that comparisons can be made between cells of different initial doubling levels. EGF was obtained from Collaborative Biomedical Products (Bedford, MA).

**Cell Proliferation Assay**—EGF-induced proliferation was determined by incorporation of [3H]thymidine by standard procedures (24). Cells were grown to confluence in 12-well plates and quiesced for 48 h in Dulbecco’s modified Eagle’s medium (DMEM) with 0.1% dialyzed FBS and then incubated with EGF (1 nm) for 16 h. [3H]Thymidine (5 μCi/well) was added, and cells were incubated for a further 10 h.

**In Vitro Wound Healing Assay**—Basal and EGF-induced migration was assessed by the ability of the cells to move into an acellular area as described previously (15). Cells were plated on a 6-well plastic dish and grown to confluence in DMEM with 7.5% FBS. After a 48-h quiescence in media with 0.1% dialyzed FBS, an area was denuded by a rubber policeman. The cells were then treated with or without EGF (1 nm; a concentration that provided maximal motility of Hs68 cells (data not shown)) and incubated at 37 °C. Photographs were taken at 0 h and 24 h, and the distance traveled by the cells at the acellular front was determined.

**Immunoblotting and Immunoprecipitation**—The levels of target molecules were assessed by immunoblotting. Cells (4 × 10⁶) were treated with EGF (1–10 nm). Cell lysates were separated on 7.5% or 10% SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to a polyvinylidene difluoride membrane (Millipore, Bedford, MA). The blot was probed with anti-EGFR antibody (ab5–104, Upstate Biotechnology Incorporated, Lake Placid, NY), anti-β-insulin receptor antibody (16630, Transduction Laboratories, Lexington, KY), anti-transferrin receptor antibody (GR09, Calbiochem), anti-α-actin antibody (A-2068, Sigma), anti-phospho-Erk-MAPK (9101, New England Biolabs, Beverly, MA), and anti-phospho-EGFR (05–104, Upstate Biotechnology Incorporated, Lake Placid, NY). Cells were then treated with or without EGF (1 nm; a concentration that provided maximal motility of Hs68 cells (data not shown)) and incubated at 37 °C. Photographs were taken at 0 h and 24 h, and the distance traveled by the cells at the acellular front was determined.
Loss of EGFR Signaling in Fibroblast Aging

The activation status of PLCγ was determined by immunoprecipitation followed by immunoblotting. Cells (2 × 10⁶) were treated with EGF as described, and lysates were incubated overnight at 4 °C with anti-PLCγ-1 antibody (05-163, Upstate Biotechnology Inc.). Immuno-complexes were captured with protein G-agarose beads and washed three times with 20 mM HEPES buffer, pH 7.4, containing 10% glycerol, 0.1% Triton X-100, 500 mM sodium chloride, 1 mM sodium vanadate. Immunoprecipitates were analyzed following immunoblotting using anti-phosphotyrosine antibody (PY-20, Transduction Laboratories). EGFR Expression Levels—The expression level of EGFR was determined by a standard binding assay (30). Cells were grown to confluence in 12-well plates and washed twice with binding buffer (DME with 1% bovine serum albumin (Fraction V, Sigma)). 0.1 nM [¹²⁵I]EGF (ICN, Irvine, CA) was added to unlabelled EGF (0–10 nM) in binding buffer. Plates were incubated for 2 h at 4 °C, and then the unbound-labeled EGF was collected. Cells were lysed with lysis buffer (Tris-buffered saline with 0.5% SDS). Both unbound and bound radioactivity was counted by γ-counter (Beckman Instruments). The number of binding sites was calculated by Scatchard analysis using linear regression.

mRNA Analyses—Northern blot analyses to quantitate message levels were performed using 3 μg of mRNA purified by TRIzol (Life Technologies, Inc.) and Oligo-(dT) cellulose (Life Technologies, Inc.). RNA was electrophoresed and transferred to nylon membrane Hybond N+ (Amersham Pharmacia Biotech) and probed according to the standard procedures. A probe for EGFR was prepared from human EGFR cDNA (15). A probe for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was prepared from the human GAPDH cDNA (ATCC). The probes were specific for phosphotyrosine (PY-20, Transduction Laboratories), phospho-Erk-MAPK (9102, New England Biolabs), or pan-Erk-MAPK (#9102, New England Biolabs). In the phospho-PLCγ analysis, cells were treated in the absence or presence of 1 nM EGF for 5 min. Cells lysates were immunoprecipitated with the anti-PLCγ antibody, size-fractionated by SDS-PAGE, and immunoblotted with anti-phosphotyrosine antibody. Shown are representative blots of at least two repeats at all data points.

Fig. 2. Tyrosyl phosphorylation of intracellular targets of EGF receptor signaling in Hs68. Cells were treated with or without 1 nM EGF for 5 min. Cells were lysed, and equal volumes of cell lysates were size-fractionated by 7.5% SDS-PAGE and immunoblotted with antibodies specific for phosphotyrosine (PY-20, Transduction Laboratories), PLCγ (#05–163, Upstate Biotechnology Inc.), phospho-Erk-MAPK (#9101, New England Biolabs), or pan-Erk-MAPK (#9102, New England Biolabs). In the phospho-PLCγ analysis, cells were treated in the absence or presence of 1 nM EGF for 5 min. Cell lysates were immunoprecipitated with the anti-PLCγ antibody, size-fractionated by SDS-PAGE, and immunoblotted with anti-phosphotyrosine antibody. Shown are representative blots of at least two repeats at all data points.

Fig. 3. EGFR expression levels in different aged cells. A, cells were grown to confluence and lysed. Cell lysates were size-fractionated by 7.5% SDS-PAGE and immunoblotted with anti-EGFR (#05–104, Upstate Biotechnology Inc.), anti-transferrin receptor (GR09, Calbiochem), anti-insulin receptor β-subunit (116630, Transduction Laboratories), or anti-α-actin antibodies (A-2066, Sigma). EGFR and other receptor expression levels were enumerated by densitometry and calculated as a ratio of α-actin. Representative blots are shown of at least two repeats experiments. The receptor expression levels were determined as the ratio to α-actin level by densitometry analysis. The data are the mean ± S.E. of more than two independent studies. Statistical analysis was performed by Student’s t test as compared with P5 of Hs68: *, p < 0.05, **, p < 0.01.

B, EGFR binding sites were enumerated by standard Scatchard analyses as described under “Experimental Procedures.” The numbers of binding sites were calculated by Scatchard plot using linear regression. Shown are representative values; all values were determined twice except for CRL-1489. The values for Hs68 are the means of more than two independent studies. ○, Hs68; ☐, cells from fetus male (CRL-1475); □, cells from 1-month-old male (CRL-1489); ×, cells from 17-year-old male (CRL-7315); ⊗, cells from 83-year-old male (CRL-7815).
brane Hybond N+ (Amersham Pharmacia Biotech) for 12 h at 42 °C. The membranes were washed and exposed for autoradiography. The amounts of gene transcription were determined by densitometry.

**Cyclic AMP (cAMP) Assay**—Cells were plated in 10-cm culture plate and grown to confluence in DME with 10% FBS. Cells were treated with interferon-γ-inducible protein-10 (50 ng/ml) (Peprotech, Rocky Hill, NJ) for 4 h or forskolin (25 μM) (Sigma) for 30 min. Ice-cold extraction buffer (50% ethanol, 0.1 N HCl) was added and incubated on ice for 15 min. Extracts were lyophilized and re-suspended in 100 μl of water. cAMP was quantitated using a cAMP assay kit (Amersham Pharmacia Biotech). After the extraction, cells were lysed with 0.1 N NaOH and was quantitated using a cAMP assay kit (Amersham Pharmacia Biochem). The data were the mean ± S.E. of at least two experiments at each point except for CRL-7815. Statistical analysis was performed by Student’s t test as compared with early passage of cells.

**Bromodeoxyuridine (BrdUrd) Staining**—Near senescent or dermal fibroblasts from different aged individuals were observed by BrdUrd staining. Cells were treated with interferon-γ-inducible protein-10 (50 ng/ml) for 4 h or forskolin (25 μM) for 30 min. Cells were fixed by 70% ethanol and stained with a BrdUrd staining kit (HCS24, Oncogene research product, Cambridge, MA).

**RESULTS**

**Aged Hs68 Present Reduced Basal and EGF-induced Mitogenic and Cell Proliferative Capacities**—Hs68 cells reproducibly senesce at P19 (n = 4). We note two separate populations in terms of EGF responsiveness: early- and mid-passage (>PDR10) and late-passage (<PDR10) (Fig. 1A). The average basal cell motility decreases steadily from 360 μm/day in early passage down to 290 μm/day in near senescent fibroblasts, although only by P18 (PDR3) was there a statistical difference in motility. EGF-induce motility was relatively constant until about P13 (PDR18) at >470 μm/day (average 1.7-fold induction); thereafter it fell precipitously to 300 μm/day by P18 (PDR3) (1.1-fold induction).

Basal thymidine incorporation remained low but steady during early- and mid-passage (average 8300 cpm) until late passage (P18, PDR3 presented 850 cpm) (Fig. 1B). EGF-induced mitogenesis was strong early (15-fold for early- and mid-passage) and decreased as cells approached senescence (0.9-fold at P18, PDR3). These data on proliferative capacities mirror earlier reports (32, 33), thus validating this use of this cell line for in vitro aging studies.

**Loss of EGFR Signaling in Fibroblast Aging**

**Expression of Exogenously encoded EGFR**—Near senescent CRL-7815 cells from an 83-year-old male donor (tested at P6; reproducibly senesced at P7) and Hs68 cells (P18, PDR3; reproducibly senesced at P19) were electroporated with a human EGFR cDNA driven from the SV-40 early promoter (15). Green fluorescence protein (GFP) plasmid (Life Technologies, Inc.) was introduced in parallel, and GFP expression was determined by fluorescence microscopy after 48 h to assess efficiency of electroporation. Approximately 10⁷ cells were electroporated (500 μg, 0.320 kV) with 20 μg of DNA in a total volume of 500 μl. Electroporated cells were incubated for 48 h in DME with 0.1% dialyzed FBS before experimentation.

**Determination of EGFR Internalization**—Cells were grown to confluence in 6-well plastic plates and washed twice with binding buffer (as described in Scatchard analyses for EGFR expression levels). Cells were pre-incubated with binding buffer for 1 h at 37 °C and incubated in 0.1 nM ¹²⁵I-EGF (ICN) for 10, 8, 6, 4, 2, and 0 min at 37 °C. Cells were washed with ice-cold binding buffer at the end of incubation. Surface bound ¹²⁵I-EGF was obtained by collecting two washes of the cells with acid strip buffer (50 mM glycine, 100 mM NaCl, 2 mg/ml polyvinylpyrrolidone, 2x x urea, pH 3.0 adjusted with HCl). Internalized ¹²⁵I-EGF were obtained by lysing the cells with 1x NaOH. Both surface and internalized radioactivity was counted by γ counter (Beckman). The endocytic rate constants were calculated by the time course of loss of surface-bound EGF and accumulation of internalized EGF (31).

**Ligand-induced internalization of EGFR in in vitro aged Hs68 fibroblasts (A) and fibroblasts from male donors (B).** Internalized and surface-bound EGF were determined using ¹²⁵I-EGF as described under “Experimental Procedures.” The endocytic rate constants were calculated by the time course of loss of surface-bound EGF and accumulation of internalized EGF. The data are the mean ± S.E. of at least two experiments at each point except for CRL-7815. Statistical analysis was performed by Student’s t test as compared with early passage of cells.

**Cells from Aged Individuals Present Lower Basal Capacities and Lose EGF Responsiveness Sooner**—To determine whether the diminutions in absolute and EGF-induced responses that occurred during in vitro aging were mimicked by in vivo aging, dermal fibroblasts from different aged individuals were ob-

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**Fig. 4.** Ligand-induced internalization of EGFR in in vitro aged Hs68 fibroblasts (A) and fibroblasts from male donors (B). Internalized and surface-bound EGF were determined using ¹²⁵I-EGF as described under “Experimental Procedures.” The endocytic rate constants were calculated by the time course of loss of surface-bound EGF and accumulation of internalized EGF. The data are the mean ± S.E. of at least two experiments at each point except for CRL-7815. Statistical analysis was performed by Student’s t test as compared with early passage of cells. ***, p < 0.01.**
blast populations, from the 1-month-old male (CRL-1489) and the 83-year-old male (CRL-7815), the cells from the aged individual senesced earlier in vitro (P7 versus P16; n = 2). The basal cell migrative and proliferative capacities gradually decreased during in vitro aging of the CRL-1489 cells down to levels comparable with the CRL-7815 cells (Fig. 1C). Late passage cells from either donor lost EGF responsiveness in cell migration (1-month-old (CRL-1489): 1.4–1.2-fold; 83-year-old (CRL-7815): 1.8–1.3-fold) and cell proliferation (CRL-1489: 4.7 to 2.3, CRL-7815: 3.1 to 1.1) compared with earlier passages of the cells (P12 of CRL-1489, P3 of CRL-7815). Thus, cells aged in vivo presented less reserve in their responsiveness to EGF, a situation that may become limiting during wound healing repopulation.

EGFR Signaling and Expression Levels Are Decreased in Aged Fibroblasts—Diminished cellular activities could be due to alterations at any intracellular level from decreased signaling to reduced end-target action. To determine the site of age-related decrease in EGF-induced responses, we assessed activation of downstream signaling pathways and receptor functioning in aged fibroblasts (Fig. 2). EGFR kinase activity, including auto-phosphorylation, and Erk1/2-MAPK tyrosyl phosphorylation, as markers of activation (24, 35), were enhanced by EGF stimulation in early passage Hs68 cells. PLCγ tyrosyl phosphorylation, a surrogate marker of activity (36), was at a high basal level in early passage Hs68 fibroblasts and was only minimally increased by EGF stimulation. In near senescent Hs68 cells (P17) EGF-induced EGFR kinase and auto-phosphorylation are reduced, and there is little if any PLCγ and MAPK tyrosyl phosphorylation. EGF-induced kinase activity and activation of PLCγ and Erk-MAPK are decreased in fibroblasts from the 83-year-old donor compared with those from the fetal or 1-month-old donors (data not shown). However, the signaling is not decreased to the same extent as the near senescent Hs68 cells; this is consistent with the cell response data. The reduced phosphorylation of PLCγ and Erk-MAPK is not due to availability of these effectors, as their levels are essentially unchanged during aging (Fig. 2).

We postulated that the reduced EGFR signaling was due to a receptor-level deficit as two divergent pathways were similarly affected, and EGFR levels were shown to be decreased in an earlier report (21). Total cellular EGFR levels were assessed using immunoblotting of whole cell lysates (Fig. 3A). EGFR levels in aged cells were down to about half in early population doubling level cells. To determine whether this represented a specific loss of EGFR or whether there was a global decrease in EGFR mRNA expression levels (A and B) and stability (C). A, B. The expression levels of EGFR were determined by Northern blot analyses. Shown are representative blots of at least two blots for each situation. C, RNAs were collected before and after 2.5 h of treatment with actinomycin D (5 μM). The amount of mRNA for EGFR and GAPDH were determined by Northern blot analyses and phosphoimaging analyzer (Bio-Rad). The data shown is the ratio to P6 of Hs68 without actinomycin D, shown in all the blots is the ~5.8 kilobase mRNA species that represented the overwhelmingly predominant band in all experiments.

Aging effects on EGFR mRNA expression levels (A and B) and stability (C). A, B. The expression levels of EGFR were determined by Northern blot analyses. Shown are representative blots of at least two blots for each situation. C, RNAs were collected before and after 2.5 h of treatment with actinomycin D (5 μM). The amount of mRNA for EGFR and GAPDH were determined by Northern blot analyses and phosphoimaging analyzer (Bio-Rad). The data shown is the ratio to P6 of Hs68 without actinomycin D, shown in all the blots is the ~5.8 kilobase mRNA species that represented the overwhelmingly predominant band in all experiments.

Aging effect on gene transcription. The transcriptional levels of EGFR and the other genes were measured by nuclear run-on assay. Intact nuclei were isolated by Nonidet P-40 buffer. 32P-labeled RNAs were extracted and hybridized with indicated dot-blotted DNA. The gene transcriptional level was determined by densitometry. The data are the mean ± S.E. of three independent studies. The data were shown as the ratio to GAPDH mRNA transcription. Statistical analysis was performed by Student’s t test as compared with P5 of Hs68: *, p < 0.05. The picture shown is a composite picture that was generated by overlaying all three experiments after adjusting the GAPDH density; in each assay EGFR transcriptional rates were significantly lower in the low PDR cells. IR, insulin receptor β-subunit; TR, transferrin receptor.

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cell surface receptors, the levels of the transferrin receptor and insulin receptor β-subunits were also assessed by immunoblotting and densitometry. These, representing two other classes of surface receptors, were relatively unchanged in aging (Fig. 3A) when compared with actin, suggesting a specific down-regulation of EGFR levels during cell aging. It was possible that the fewer EGFR were differentially presented, so we assessed the number of binding sites (Fig. 3B). EGFR binding sites decreased during aging down to 40% that of the level of early- and mid-passage cells (Fig. 3B). The loss of EGFR on the surface mirrored the decrease in total cell EGFR (Fig. 3A).

**EGFR Synthesis Is Decreased in Aged Cells**—The decline in EGFR may be due to increased turnover or decreased synthesis. Upon binding, ligand EGFR are rapidly internalized and degraded by a saturable pathway; therefore, we examined internalization of EGFR. Internalization was significantly decreased in aged Hs68 and cells from aged individuals (Fig. 4) in agreement with an earlier report (21). The internalization of EGFR in near senescent Hs68 (P18, PDR3) was 29% that of young Hs68 (P4, PDR45) and, in the cells from the 83-year-old male, was 44% that of the cells from the 1-month-old male. Thus, increased degradation of EGFR was unlikely to be the cause of decreased levels; rather, the decreased internalization would maintain higher levels of accessible, signaling EGFR. To investigate the effects of cell aging on production of EGFR, mRNA levels were determined (Fig. 5). In near-senescent cells, EGFR mRNA levels were significantly reduced compared with expression of a “housekeeping” gene, GAPDH. The EGFR mRNA expression level of near senescent Hs68 (P18, PDR3) was barely detectable (Fig. 5A), as was the EGFR mRNA expression level of cells from the 83-year-old male (Fig. 5B).

We also assessed the effect of aging on EGFR mRNA stability. The mRNA transcription inhibitor actinomycin D treatment caused a 19% and 15% decrease of EGFR mRNA on P6 (PDR39) and P18 (PDR3), respectively (Fig. 5C). As the near senescent cells do not display greater instability than the early passage cells, cell aging does not seem to affect on EGFR mRNA stability. Transcriptional activity of EGFR mRNA was assessed by nuclear run-on assay. EGFR mRNA transcriptional level was decreased to ~50% in aged Hs68 (Fig. 6). The correlation of reduced mRNA and protein levels suggest that the age-related loss in EGFR is affected at the RNA transcription level.

**cAMP Signaling Is Relatively Maintained in Aging Fibroblasts**—To confirm that some of the signaling pathways from the cell surface are maintained in aging, we determined cAMP generation in response to a pharmacological and a physiological agent. Forskolin induced a robust increase in intracellular cAMP in both young (2.5-fold in PDR42) and near senescent Hs68 cells (2.3-fold in PDR3) (Fig. 7). The anti-motility EGF-negative CXC chemokine interferon-γ-inducible protein-10 (IP-10) also elicited a strong response in both young and near senescent Hs68 cells (2.0-fold and 1.8-fold, respectively). Thus, there does not appear to be a global decrease in all signaling pathways from the cell surface.

**EGFR Re-expression Restores the EGF-induced Cell Migration and Proliferative Capacities**—We hypothesized that decreased EGFR levels are cause for loss of EGF-induced responses in near senescent fibroblasts. The foregoing data could simply reflect a linked but not causal phenomenon of growth factor receptors decreasing during cell aging. To test this, near senescent Hs68 cells (P18, PDR3) were electroporated with cDNA encoding either EGFR or GFP (as control). About 44% of the GFP electroporated cells expressed GFP after 48 h. Despite the only partial transfection, the EGFR expression level of EGFR plasmid-targeted cells was about twice that of GFP control cells (Fig. 8), suggesting that the transfected cells expressed EGFR near the level of non-aged fibroblasts. The EGFR plasmid-targeted cells presented more EGF binding sites, as determined by Scatchard analysis (6.3 × 10^8/cell compared with 3.4 × 10^8/cell), in parallel with total EGFR levels as determined by immunoblotting and densitometry analysis (1.8-fold; Fig. 8D). These cells recovered, at least in part, the EGF-induced motility and proliferation responses compared with GFP cells (motility response increased from 1.1-fold to 2.0-fold and mitogenic response from 1.7-fold to 2.4-fold; n = 3). The lack of a complete recovery may be due to the only partial electroporation combined with the transient nature of EGFR expression. This recovery of responsiveness was also observed near senescent (P6, PDR3) fibroblasts from the 83-year-old male donor (CRL-7815). 40% of the GFP cells expressed after 48 h. These cells demonstrated recovery of EGF-induced responsiveness in both cell motility and proliferation (motility response increased from 1.1-fold to 1.4-fold; mitogenic response increased from 1.3-fold to 1.5-fold; n = 3). To better determine that the cells expressing higher, “young” levels of EGFR contributed to the EGF responsiveness, we attempted to evaluate single cells. Near senescent cells were co-electroporated with EGFR and GFP plasmid (DNA molar ratio 7.5:1); GFP-positive cells were presumed to express the exogenous EGFR. GFP-expressing cells were over-represented in the cells that migrated from the denuded front (68% of migrating cells versus 40% in the population) and appeared to migrate further. BrdUrd staining was performed to access proliferative activity. Cells were electroporated with or without EGFR (20 µg) and GFP (20 µg). GFP-positive cells were marked by BrdUrd incorporation at 1.5 times the fraction as GFP-negative cells (1.46- and 1.49-fold, n = 2); this ratio is similar to the increase in thymidine incorporation noted in mass cultures. The results of the single cell analyses mirror the population studies and strongly suggest that the restoration of EGF responsiveness was due to the EGFR-re-expressing cells.

**Increased EGFR Signaling Capacity Does Not Enhance the Cell Migrative Activity in Young Cells**—Our finding that re-expression of EGFR in near senescence cells restored EGF...
responsiveness could have been due to a simple dose-response effect if responsiveness was linearly related to the EGFR level. Thus, we assessed the effect of increased EGFR signaling capacity in young cells. Early passage of HS68 (P5, PDR42) was electroporated with EGFR or GFP (as control). About 45% of GFP electroporated cells expressed GFP after 48 h. Although EGFR electroporated cells expressed more EGFR, these cells did not increase EGF-induced cell migrative capacity compared with GFP cells (Fig. 9, A and B). Furthermore, electroporation of excessive EGFR cDNA led to ligand-induced cytotoxicity, as described in the literature for supraphysiological levels of EGFR (38). A second method for increasing EGFR signaling would be to use supersaturating concentrations of ligand (10 nM) in lieu of our usual dose that approximates $K_d$ (1 nM). Increasing the EGF concentration 10-fold had no effect on the response of near senescent cells. Early passage cells did not demonstrate increased responsiveness to high levels of ligand (Fig. 9C). Thus, merely increasing EGFR signaling capacity in these cells does not lead to increased responsiveness, suggesting a qualitatively distinct mechanism for loss of responsiveness.

**DISCUSSION**

The data presented herein suggest a model of dermal fibroblast aging in which both basal and EGF-stimulated cell activities decline with decreasing PDR. The loss of EGFR responsiveness in near senescent cells is linked to decreased level of EGFR production. The decrease in basal cell activities is likely due to a second, possibly independent cell change that does not involve EGFR signaling. It remains to be determined whether these two events have a common causal basis at the gene regulation level.

During in vitro aging, the decline in EGFR was reflected by loss of EGFR responsiveness for both migration and proliferation responses. This occurred most acutely in near senescent fibroblasts. The loss of two distinct and competing cell responses (14, 24), along with decreased activation of two divergent intracellular signaling pathways despite unchanged levels of protein, suggested that the reduced EGFR was cause for the response deficit.

Age-related diminution in non-stimulated dermal fibroblast motility has been attributed to global cell changes (23, 39, 40) and would thus be independent from the loss of EGF responsiveness noted in near senescent cells. This fits with our findings on early passage fibroblasts from aged donors; basal motility decreases, whereas EGF responsiveness is retained despite declining levels of EGFR. On the other hand, autocrine growth factor signaling has been proposed as stimulating dermal fibroblast (41), although this has yet to be documented for EGF signaling (42), raising the possibility that the EGFR decreases underlies both phenomena. At present, we cannot eliminate the possibility that some level of autocrine signaling through EGFR occurs in these fibroblasts and that the decreasing motility and mitogenesis noted in the absence of exogenously added EGF reflects a parallel decrement in this EGFR-mediated signaling (43). In fact, the unstimulated levels of PLC and Erk-MAPK activation are high compared with mouse immortalized fibroblasts (24) and do decline in parallel with age-related reduction in EGFR. Still, we do not favor this latter scenario, since the early passage dermal fibroblasts from different aged donors all present EGF-induced motility despite significantly reduced EGFR levels on the cells from the aged donors.

We found that EGFR levels preferentially declined during cellular aging regardless of whether the cellular aging occurred in vivo and in vitro. This was noted as loss in ligand binding sites per cell and as total cellular EGFR protein. Thus, this loss of responsiveness does not merely reflect altered trafficking, delayed glycoprotein maturation and transit, or increased endocytosis and retention. We investigated the underlying mechanism for decreased EGFR. Paradoxically, the data show that receptor internalization is diminished with aging, as previously reported (21), as if to spare the few receptors from down-regulation since signaling from the surface is sufficient to elicit both the biochemical and physiological events leading to the cell responses of mitogenesis and motility (44, 45). On the other hand, EGFR mRNA levels were diminished in parallel with reduced surface and total EGFR. That this is at least semi-specific for EGFR is demonstrated by comparing EGFR protein levels to the cytoskeletal protein actin and two cell surface receptors, the receptors for insulin and transferrin. These lat-
ter represent two biologically and biochemically distinct receptor classes. Although an age-related transcriptional control element remains to be identified, the results of our nuclear run-on experiment suggest that this preferential loss of EGFR seems to be caused by the decrement of mRNA transcriptional activity.

In addition to EGFR, other growth factor receptors are also decreased in aging cells; these include receptors for fibroblast growth factor and platelet-derived growth factor (22, 23, 43, 46). It is possible that the diminished levels of these might be controlled by similar mechanism as EGFR. Our data show EGFR expression levels parallel PDR number, not necessarily donor age, as might be expected from earlier reports (34, 47). In addition, the PLCy and Erk1/2-MAPKs, representing two signaling pathways that diverge from the receptor (24), also were relatively unchanged. These observations do not eliminate the possibility of other motility-signaling molecules also being specifically down-regulated, especially in other cell types, as might be the situation for aged hepatocytes that appear to be deficient in EGF coupling to Shc (48). Thus, our findings do highlight EGFR expression as a marker of age-related alterations in the fibroblast cell proteome.

These data show that decreased functioning in aged cells is due not to a global deficit but to specific deficits. Signaling from some cell surface receptors is decreased in aging (46), and this decrease is likely controlled separately from global cell dysfunction. To support this, we found that insulin receptor levels were relatively unchanged in near senescent Hs68 cells and, in parallel, the insulin responsiveness in cell migrative and cell proliferative capacities were relatively unchanged. However, insulin had very little effect on cell migrative and cell proliferative capacities compared with EGF in Hs68 fibroblast (data not shown). To confirm that some signaling pathways from cell surface receptors were maintained in the face of near senescent function, we assessed the effect of forskolin and interferon-γ-inducible protein-10 on the generation of intra-cellular cAMP (37). The near senescent cells responded similarly to the young Hs68 cells. Thus, at least some signaling pathways are intact in aged cells.

Decreased levels of EGFR appeared to be the underlying mechanism for loss of responsiveness to EGF in aged cells. As this was reflected as diminished transcription and not increased consumption, we could correct this deficit by introducing EGFR cDNA under an autonomous promoter. Aged cells were successfully electroporated to express EGFR levels similar to "young" cells; these cells regained their EGFR responsiveness. These experiments demonstrate for the first time that specific cellular response deficits to factors in near senescent cells can be corrected by restoring a single gene product. Although not advocating such an approach, these data support the use of EGFR gene transfer to nonhealing wounds as a more rational therapy than application of growth factors, since the rate-limiting step is receptor signaling and not factor availability (although specific growth factors are reduced in wounds in aged patients (43)). Furthermore, we do not know whether introduction of EGFR into senescent cells will restore any responses, as we were unable to stably introduce EGFR cDNA either under constitutive or inducible transcriptional controls. In fact, senescence is likely independently regulated from EGFR levels and responsiveness. We and others (20, 21, 49, 50) also noted an age-related decrease in basal cell mitogenesis and motility. Whether this was related to the loss of EGFR signaling could be tested in immortalized human fibroblasts that had been rescued from senescence by stable expression of telomerase (51). We determined that these cells had not regained the EGF responsiveness associated with non-senescent dermal fibroblasts by comparing these cells to late passage controls. However, this was confounded by finding that average basal cell motility and thymidine incorporation was higher in two different human telomerase-positive cell populations (510 μm/ day compared with 310 μm/day and 9200 cpm compared with 2900 cpm). The EGFR responsiveness of the immortalized cells was, if anything, reduced when compared with the two aged control fibroblast populations (1.3-fold compared with 2.6-fold).

This may reflect the fact that the expression levels of EGFR in human telomerase-positive cells were not higher, and possibly lower, than in the human telomerase negative control cells. These data point to the multiple, possibly independently regulated alterations during aging that lead to diminished cell activities. Thus, our model remains one of a global cell alteration reducing cell responsiveness gradually with aging, with an acute loss of EGF responsiveness superimposed as the cells approach senescence.

EGFR re-expressed cells recover robust EGF responsiveness,
although the EGF-induced cell mitogenesis is still lower than that of early passage cells or cells from neonatal donors. The partial recovery of cell responses may be due to incomplete electroporation efficiency combined with the transient expression or to age-related decrements in general cell functioning such as protein synthesis (52–55), which is involved in cell proliferation or to age-related decrements in general cell functioning.

The implications are that interventions to improve healing in aged skin need to be targeted toward improving cell responsiveness by enhancing cell signaling and augmenting the cell responses.

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