Supporting Information

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SI Materials and Methods

Plasmid Construction. Codon-optimized human CXCR4 cDNA (1) was kindly provided by Joseph G. Sodroski (Dana-Farber Cancer Institute, Harvard University, Boston) and cloned into the pBabe retroviral vector. A constitutively active form of swine TGF-β1 cDNA vector, pPK9a (2), was kindly provided by Lalage M. Wakefield (National Cancer Institute, Bethesda, MD) and cloned into the pBabe-neo retroviral vector. The shRNA oligonucleotides against GFP, CXCR4, SDF-1, p53, TβRII, and Smad4 genes were generated and cloned into lentivirus-derived pLKO-hygro- or pLKO-puro-vectors (3). Target sequences used are listed in the table below.

Isolation of Human Mammary Fibroblasts and Tissue Culture. Normal human stromal fibroblasts were extracted from healthy breast tissue obtained from a reduction mammoplasty as described previously (4) and cultured in DMEM supplemented with 10% fetal bovine serum (Valley Biomedical). The retroviral pMIG (MSCV-IRES-GFP) vector, expressing both hTERT and GFP, and a pBabe-puro vector encoding a puromycin resistance gene, were infected into these mammary fibroblasts to facilitate their immortalization. Mammary fibroblasts were also extracted from tumor masses or noncancerous breast tissues obtained from breast cancer patients, as described previously (4).

Subcutaneous Tumorigenicity Assays. MCF-7-ras human breast carcinoma cells (1 × 10⁶) and human mammary fibroblasts (3 × 10⁶) were admixed and suspended in 400 μL of Matrigel (BD Biosciences). The mixture was injected s.c. into immunodeficient nude mice. Tumorigenic assay was performed as described previously (5).

Evaluation of Angiogenesis in MCF-7-ras Tumor Xenografts. Serial paraffin sections (taken at 2-mm intervals) were prepared from tumor xenografts grown. A total of 30 sections, six independent tumors from each cohort, were immunostained using an anti-CD31 antibody, a marker of vascular endothelial cells. Microvessel density was assessed as previously described (6).

Retroviral and Lentiviral Infections. Retroviral and lentivirus infections were performed as described previously (3, 5). After infection, human mammary fibroblasts were cultured for 4–6 d in the presence of the appropriate antibiotic for each plasmid: puromycin (1 μg/mL), neomycin (500 μg/mL), hygromycin (50 μg/mL), or blasticidin (7.5 μg/mL).

Western Blot Analysis. Fibroblasts were seeded at 0.5 × 10⁶ cells per 6-cm dish and cultured for 48 h in DMEM supplemented with 2% calf serum. A total of 10–30 μg of whole-cell lysate was run on NuPAGE 4–12% gradient gels (Invitrogen) and transferred onto Hybond ECL membrane (GE Healthcare). Quantification of band intensity was performed using Multi Gauge Version 2.2 software (Fujifilm).

Real-Time RT-PCR Analysis. Total RNA was extracted using an RNeasy Plus Mini Kit (Qiagen) in accordance with the manufacturer’s protocol. SuperScript II reverse transcriptase (Invitrogen) was used to synthesize cDNA. Real-time RT-PCR analysis was performed as previously described (7). Data for each sample were normalized relative to the expression level of β2-microglobulin gene. Primers used for RT-PCR analyses are described in a table below.

Flow Cytometry. 1–5 × 10⁵ human mammary fibroblasts were analyzed using FACSCalibur flow cytometry (Becton Dickinson) as previously described (4). The antibody used to detect CXCR4 is listed in a table below. Nonviable cells were detected by staining with 7-aminoactinomycin D (7-AAD; BD Biosciences).

Measurement of SDF-1 Protein Levels. Various human mammary fibroblasts were cultured in DMEM with 2% serum for 48 h. The media conditioned by fibroblasts were collected and filtered through a 0.45-μm syringe filter. SDF-1 levels were measured using the Quantikine human SDF-1 immunoassay (R&D Systems) in accordance with the manufacturer’s protocol.

Measurement of Levels of Biologically Active TGF-β1. Levels of active TGF-β1 were measured using mink lung epithelial cells (MLECs) expressing a PAI-1 promoter-driven luciferase reporter construct (8), a kind gift from Daniel B. Rifkin (New York University Medical Center, New York). Cells (1.6 × 10⁴) were seeded onto a 96-well plate and incubated for 14 h in media conditioned by exp-CAF5 or the control fibroblasts for 2 d. Luciferase activity was measured using the Dual-Luciferase Reporter Assay System (Promega) in accordance with the manufacturer’s protocol.

Immunostaining of Human Breast Tissues and Cultured Mammary Fibroblasts. Mammary fibroblasts were stained using various antibodies as described below. To quantify α-SMA-positive cells (%), the positive cell numbers relative to total cell numbers (>100 counted cells) were evaluated in nine independent fields from three different wells of each fibroblast type under a fluorescence microscope. Paraffin sections prepared from human breast tissues were immunostained using either the Dako EnVision system (DAKO) or the avidin-biotin complex technique. The antibodies used are listed in the table below.

Cell Cycle Analysis. Cell cycle analysis using propidium iodide was performed as described previously (9). Different fibroblast populations were exposed to 20 Gy of ionizing radiation (137Cs) and harvested 30 h after irradiation for analysis.

Statistical Analysis. Statistical analyses were performed using a Student t test or ANOVA test, followed by a Dunnett’s multiple comparison test using the SPSS version 13.0 software. Values of P < 0.05 were considered significant.

Antibodies and Chemicals. Primary antibodies used are listed in the following table. AMD3100 octahydrochloride hydrate and SB431542 were purchased from Sigma-Aldrich. Recombinant SDF-1α and TGF-β1 proteins were obtained from R&D Systems.
Antibodies (clone name) | Source | Purpose
--- | --- | ---
β-actin (8226) | Abcam | WB
CD31 | Santa Cruz Biotechnology | IHC
Collagen type1 1A | Sigma | IF
Pan-cytokeratin | Sigma | IF
CXCR4 (12G5) | R&D Systems | IHC
CXCR4-biotin (12G5) | R&D Systems | FCM
CXCR4-PE (12G5) | R&D Systems | FCM, IF
CXCR4 | Leinco Technologies | WB
Fibroblast surface protein | Abcam | IF
Fibronectin | BD Biosciences | IF
S100A4/FSP-1 | Dako | IF
SDF-1 (K15C) | Arenzana-Seisdedos* | IHC
α-SMA (1A4) | Sigma | IF, WB, IHC
α-SMA-Cy3 (1A4) | Sigma | IF
Smad2/3 | BD Biosciences | IF, WB
Phosphorylated-Smad2 (138D4) | Cell Signaling Technology | WB, IHC
Smad4 (B-8) | Santa Cruz Biotechnology | WB
p53 | Santa Cruz Biotechnology | WB
Prolyl-4-hydroxylase (5B5) | Oncogene | WB
pan-Ras (Ab-1) | BD Biosciences | IF
TGF-β RIIb | R&D Systems | WB
Tenascin C | Gift from Luciano Zardi† | IF
α-tubulin (DM1A) | Abcam | WB
Vimentin (V9) | Novocastra Laboratories | IF

FCM, flow cytometry; IF, immunofluorescence; IHC, immunohistochemistry; WB, Western blotting.

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shRNA target sequences

| Sequence name | Sense sequence | Antisense sequence | Ref.
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GFP-shRNA | 5′-GCAAGCTGACCGTGGAGTTCA-3′ | (4) |  
CXCR4-shRNA1 | 5′-ATGATGGTGTACCTGGTCA-3′ |  
CXCR4-shRNA2 | 5′-ATCTGGGCTTCCATGGCTCA-3′ |  
SDF-1-shRNA1 | 5′-TCCTGGTGTACCGGCTTC-3′ |  
SDF-1-shRNA2 | 5′-AGCTATTACTTCCTCCC-3′ |  
TjIR1-shRNA1 | 5′-GAATGACGAGAATACACCT-3′ | (12) |  
TjIR1-shRNA2 | 5′-GATTCAGAGATTTCACTT-3′ | (13) |  
Smad4-shRNA1 | 5′-TCATCTGAGATGGCTCA-3′ | (14) |  
Smad4-shRNA2 | 5′-AGGGTGCAGTGGAATGTA-3′ | (15) |  
p53-shRNA | 5′-GACTCCAGTGTAATCTAC-3′ | (16) |  

PCR primers

| Gene name | Sense sequence | Antisense sequence |
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α-SMA | 5′-GTGCTGACAATGCGCCTC-3′ | 5′-AAGCAGCACCTGGGCT-3′ |
SDF-1 | 5′-CATGGCTGCAATGCGCCTC-3′ | 5′-GGATGCGTCCCTCCT-3′ |
TGF-β1 | 5′-AGGGTGCAGTGGAATGTA-3′ | 5′-GCTGTCACGCTCGGCT-3′ |
TGF-β2 | 5′-AGGGTGCAGTGGAATGTA-3′ | 5′-GCTGTCACGCTCGGCT-3′ |
TGF-β3 | 5′-AGGGTGCAGTGGAATGTA-3′ | 5′-GCTGTCACGCTCGGCT-3′ |
β2-microglobulin | 5′-GTGCTGACAATGCGCCTC-3′ | 5′-AAGCAGCACCTGGGCT-3′ |
v-H-ras | 5′-GTGCTGACAATGCGCCTC-3′ | 5′-AAGCAGCACCTGGGCT-3′ |
CXCR4 | 5′-GTGCTGACAATGCGCCTC-3′ | 5′-AAGCAGCACCTGGGCT-3′ |
GAPDH | 5′-GTGCTGACAATGCGCCTC-3′ | 5′-AAGCAGCACCTGGGCT-3′ |
**Fig. S1.** (A) Isolation of control fibroblasts. As a control, normal GFP-labeled, puromycin-resistant, immortalized human mammary stromal fibroblasts were injected s.c. into nude mice as pure cultures without MCF-7-ras cells. Clusters of fibroblasts, which survived at the site of injection for 42 d after implantation, were dissected, dissociated, and cultured in puromycin-containing media. The resulting puromycin-resistant cells were termed control fibroblast-1 cells. These cells were once again injected alone s.c. into nude mice for an additional 200 d. The puromycin-resistant cells were similarly extracted and termed control fibroblast-2 cells (242 d old). (B) The mesenchymal nature and human origin of the exp-CAFs and control fibroblasts. Immunofluorescence analysis of control fibroblast-2 (control f.) and exp-CAF2 cells showed intense positive staining for mesenchymal markers, including human (but not mouse) vimentin, prolyl-4-hydroxylase, collagen 1A, fibronectin, S100A4/FSP-1, and fibroblast surface protein. In contrast, the epithelial marker pan-cytokeratin was not detected in these cells. Collectively, these data indicate the human origin and mesenchymal nature of exp-CAF2 and control fibroblast-2 cells. GFP fluorescence of exp-CAF2 cells was also shown. Cell nuclei are stained with DAPI (blue). (Scale bar, 50 μm.) (C) Stable expression of α-SMA in four independent exp-CAF2 cells. Western blot analysis of fibroblasts using an anti-α-SMA antibody. The membrane stripped was reprobed by an anti-β-actin antibody. (D) No gene transfer of the oncogenic ras into exp-CAFs in vivo. The set of PCR primers permitted only the specific amplification of DNA fragments containing two mutations present in the oncogenic v-H-ras gene expressing in MCF-7-ras cells. Genomic- and RT-PCR analyses using these primers failed to detect the v-H-ras allele in the genomic DNA and cDNA of either control fibroblast-2 (control f.), 42-d-old exp-CAF1, or exp-CAF2 cells, whereas the v-H-ras allele was readily detected in the DNA of MCF-7-ras cells. Primers to amplify glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were also used as an internal control. In addition, an anti-pan-Ras antibody that recognizes Arg12 mutant forms of the various Ras oncoproteins but not wild-type Ras Gly-12 proteins, readily detected the v-H-Ras Arg-12 protein expressed by MCF-7-ras cells. However, this antibody failed to detect such protein in either fibroblast population. The membrane treated with the anti-pan-Ras antibody was also probed by an anti-α-actin antibody. (E) Increased neoangiogenesis in the exp-CAF2-containing tumors. *P < 0.05. Error bars, SE. Staining of sections prepared from tumor xenografts admixed with exp-CAF2 (a), 42-d-old exp-CAF1 (b), or control fibroblast-2 cells (c) by Masson’s trichrome, staining collagen in blue and microvessels. Immunostaining of an exp-CAF2 cell-containing tumor section was performed using an anti-CD31 antibody (d, red). GFP fluorescence of exp-CAF2 (e, green) and control fibroblast-2 cells (f, green) shown in the advanced tumors. Cell nuclei stained with DAPI (d–f, blue). (Scale bar, 100 μm.)
Fig. S2. (A) No significant up-regulation of TGF-β3 expression in exp-CAFs. Real-time PCR analysis of fibroblasts indicates fold induction of TGF-β3 expression relative to β2-microglobulin (β2-MG) expression. (B) Significant inhibition of the TGF-β type II receptor (TβRII) and Smad4 protein expression in human normal mammary fibroblasts using lentiviral shRNAs. (a) Western blot analysis using an anti-TβRII antibody showed that two different shRNAs (1 or 2) each suppressed TβRII expression by 50% or 96%, respectively, in mammary fibroblasts relative to the control GFP-shRNA. Each TβRII-shRNA also attenuated the expression level of TGF-β1-induced phosphorylated Smad2 (pSmad2) by 85% and 48% compared with GFP-shRNA. The membrane treated with an anti-pSmad2 antibody was reprobed using anti-TβRII and anti-α-actin antibodies. (b) Two different Smad4-shRNA lentivirus vectors (1 or 2) each inhibited the protein expression by nearly 100%. Western blotting of human mammary fibroblasts using an anti-Smad4 antibody. The membrane was also probed with an anti-α-tubulin antibody. (C) Activation of TGF-β signaling induces α-SMA and TGF-β expression in human mammary fibroblasts. (a) Human normal mammary fibroblasts (normal f.), forced to express a constitutively active form of TGF-β1 cDNA (active TGF-β1), exhibited elevated levels of pSmad2 (188-fold) and α-SMA (37.2-fold) protein expression compared with the control GFP-expressing fibroblasts. The membrane for immunoblotting was probed using different antibodies against pSmad2, α-SMA, Smad2/3, and α-tubulin. (b and c) Human normal mammary fibroblasts exposed to recombinant TGF-β1 (10 ng/mL) for 24 h or expressing the constitutively active TGF-β1 construct exhibited elevated levels of TGF-β1 (3.0- or 3.6-fold, respectively) and TGF-β2 (2.1- or 1.8-fold) mRNA expression compared with cells treated with PBS or expressing control GFP. (d) Real-time PCR analysis showed that TGF-β1 and -β2 mRNA expression induced by TGF-β1 treatment (10 ng/mL) for 24 h in control GFP-shRNA-expressing fibroblasts was suppressed by TβRII- (by 40% and 25%, respectively) or Smad4- (by 31–55% and 33–44%, respectively)-shRNA. These observations indicate that TβRII-Smad signaling mediates the TGF-β-induced α-SMA and TGF-β expression in mammary fibroblasts.
Fig. S3. (A) Elevated levels of SDF-1 protein expression in exp-CAFs during tumor progression. SDF-1 protein concentration in the media conditioned by fibroblasts was measured by ELISA. Error bars, SE. (B) Inhibition of SDF-1 production by exp-CAF2 cells using lentiviral shRNA. An ELISA showed that SDF-1 synthesis is attenuated by 76% or 72% in exp-CAF2 cells expressing SDF-1-shRNA-1 or -2, respectively, compared with cells expressing the GFP-shRNA. Error bars, SE. (C) Down-regulation of α-SMA expression by suppression of SDF-1-CXCR4 signaling. Immunofluorescence of exp-CAF2 cells treated with GFP- (a), SDF-1- (b and c), and CXCR4- (d and e) shRNA lentiviruses, or AMD3100, a CXCR4 inhibitor (f) using an anti-α-SMA antibody (red). (Scale bar, 50 μm.) (D) Elevated levels of CXCR4 protein expression in fibroblasts forced to express CXCR4. (a) Immunofluorescence using an anti-CXCR4 antibody (red) showed intense positive staining for CXCR4 within the cytoplasm of human mammary fibroblasts expressing a retroviral CXCR4 expression construct but not in control GFP-expressing fibroblasts. (Scale bar, 50 μm.) (b) Flow cytometry analysis to detect the cell-surface CXCR4 showed a far higher proportion (~93%) of CXCR4-positive cells in the CXCR4-expressing fibroblasts compared with the proportion (~1%) in the control GFP-expressing fibroblasts. (E) SDF-1 expression depends on activation of CXCR4 signaling in exp-CAFs. (a) Real-time PCR analysis showed that inhibition of CXCR4 expression by CXCR4-shRNA-1 or -2 decreases SDF-1 expression level in exp-CAF2 cells by 44% or 79%, respectively, compared with the GFP-shRNA. Error bars, SE. (b) Exposure of CXCR4-expressing fibroblasts to SDF-1 (100 ng/mL) for 24 h resulted in a 9.3-fold up-regulation of endogenous SDF-1 mRNA expression compared with control GFP-expressing fibroblasts treated with PBS. (F) SDF-1-CXCR4 signaling mediating an ability of stromal fibroblasts to promote tumor growth and neoangiogenesis. Exp-CAF2 or control fibroblast-2 cells expressing CXCR4- or GFP-shRNAs were mixed with MCF-7-ras human breast carcinoma cells. These mixtures were s.c. injected into nude mice. (a) Neoangiogenesis was evaluated 18 wk after injection. *P < 0.05. Error bars, SE. (b) MCF-7-ras cells were injected alone or coinjected with either CXCR4- or control GFP-expressing human mammary fibroblasts s.c. into nude mice. Tumor volumes over time are shown.
Fig. S4. (A) Induction of CXCR4 expression by TGF-β1 in human mammary fibroblasts. RT-PCR analysis of TGF-β1-treated mammary fibroblasts detecting CXCR4 and β2-microglobulin (β2-MG) expression. (B) SDF-1 expression induced by TGF-β is mediated through TβRII-Smad signaling. Real-time PCR analysis showed that SDF-1 expression induced by TGF-β1 (10 ng/mL) for 24 h in normal mammary fibroblasts expressing GFP-shRNA, was attenuated in cells expressing TβRII- (by 70%) or Smad4- (by 62–70%) shRNA. (C) TGF-β-induced CXCR4 expression is independent of the Smad signaling pathway. RT-PCR analysis of TGF-β1-treated mammary fibroblasts detecting CXCR4 and β2-MG expression. Inhibition of Smad signaling by Smad4-shRNA-1 or -2 failed to suppress CXCR4 expression induced by treatment with TGF-β1 (10 ng/mL) for 24 h in human mammary fibroblasts compared with the effect of GFP-shRNA. (D) Induction of CXCR4 signaling elevates TGF-β expression in mammary fibroblasts. Real-time PCR analysis was performed using primers specific to TGF-β1 and 2. Either control GFP- or CXCR4-expressing human mammary fibroblasts were cultured in the presence or absence of SDF-1 protein (100 ng/mL). Exposure of CXCR4-expressing fibroblasts to recombinant SDF-1 protein (100 ng/mL) for 24 h resulted in up-regulation of endogenous TGF-β1 (4.4-fold) and TGF-β2 (4.2-fold) mRNA expression compared with control GFP-expressing fibroblasts cultured without SDF-1.

Fig. S5. α-SMA, CXCR4, phosphorylated Smad2, and SDF-1 proteins are not detected in stromal fibroblasts in normal breast tissue. Immunohistochemistry of sections prepared from the normal human breast tissue using antibodies against α-SMA (A, green), CXCR4 (A, red), and pSmad2 (B, brown). Sections were also stained with DAPI (A, blue) or hematoxylin (B, pale blue). Stromal fibroblast-like cells staining negative for α-SMA/CXCR4 (A) or pSmad2 (B) are highlighted by arrows. Arrowheads depict α-SMA-positive myoepithelial cells (A, green). Normal histology of mammary gland is indicated by asterisks. (Scale bar, 50 μm.) We have previously observed that stromal fibroblasts in the normal human breast tissue are negative for SDF-1 (4).
Fig. S6. (A) CAFs prepared from breast cancer patients show elevated levels of SDF-1 and TGF-β expression. Primary stromal mammary fibroblasts were isolated from three different patients with invasive ductal breast cancer. CAFs (designated CAF1, CAF3, and CAF6) were extracted from the tumor mass, and counterpart fibroblasts (counter.f.1, counter.f.3, and counter.f.6) were isolated from the noncancerous breast stroma of the same individual as a patient-specific control. The tumor-promoting ability of these CAFs has been confirmed in our previous work (4). Real-time PCR analysis and a luciferase assay showed increased levels of SDF-1, TGF-β2, and active TGF-β expression in CAF1 (3.2-, 2.3-, and 1.6-fold, respectively), CAF3 (2.2-, 1.2-, and 0.8-fold), and CAF6 (4.4-, 2.7-, and 6.1-fold) cell populations in comparison with their control counterpart fibroblasts. Error bars, SE. 

(B) Autocrine signaling loops mediated by SDF-1 and TGF-β operate in the patient-derived CAFs in both self-stimulating and cross-communicating fashions. 

(a) Real-time PCR analysis showed that inhibition of SDF-1 or TGF-β signaling by SDF-1-, CXCR4-, TβRII-, and Smad4-shRNA in the patient-derived CAF1 cells suppressed the expression levels of SDF-1 (by 50–60%, 54–60%, 88%, or 86–94%, respectively), TGF-β2 (50–67%, 61–69%, 48%, or 69–82%), and α-SMA (77–84%, 77–85%, 49%, or 73–85%) compared with the effect of the control GFP-shRNA. (b) α-SMA-positive cells (%) were measured by immunofluorescence analysis using an anti-α-SMA antibody. Inhibition of SDF-1 or TGF-β signaling by SDF-1-, CXCR4-, TβRII-, or Smad4-shRNA in the patient-derived CAF1 cells decreased the proportion of α-SMA-positive cells (by 92–98%, 94–94%, 38%, or 46–99%, respectively) compared with the control GFP-shRNA. Error bars, SE.
Both exp-CAFs and patient-derived CAFs maintain intact p53 function. To find signs of p53 alteration in exp-CAFs, we undertook the following experiments. For example, cells bearing mutant p53 alleles almost invariably express elevated levels of this protein (17) and fail to induce p21 protein that is a well-known downstream target gene (18). (A and B) However, Western blot analysis using an anti-p53 antibody showed that exp-CAF2 cells express an equivalent level of p53 protein compared with the control fibroblast-2 and 42-d-old exp-CAF1 cells, and that the p53 gene was intact, because the expression could be induced 10 h after exposure to 20 Gy r-irradiation. Control fibroblast-2 and exp-CAF2 cells expressing the GFP-shRNA also showed normal p53 function, as they yielded significantly induction of p21 protein following r-irradiation, whereas p53-shRNA-expressing fibroblasts failed to do so. The membranes treated with an anti-p53 antibody were reprobed by antibodies against p21 and/or α-tubulin. (C) Patient-derived CAF1, 2, 3, and 6 cells (4) showed strong induction of p21 and p53 protein expression 10 h after exposure to 20 Gy r-irradiation. Immunoblotting using antibodies against p53, p21, and α-tubulin. (D) Flow cytometry analysis showed that both exp-CAF2 cells and patient-derived CAF1 cells exhibit sustained G1 arrest 30 h after exposure to 20 Gy r-irradiation. This was comparable to their corresponding control fibroblasts, whereas p53-null mouse embryonic fibroblasts (MEFs) failed to maintain cells in G1 arrest. Cell proportions (%) in G1 and G2/M are indicated. These observations strongly suggest that alteration in p53 signaling is not responsible for the induction or maintenance of TGF-β and SDF-1 autocrine signaling in tumor-promoting CAF myofibroblasts.