Autocrine TGF-β and stromal cell-derived factor-1 (SDF-1) signaling drives the evolution of tumor-promoting mammary stromal myofibroblasts

Yasushi Kojima*, Ahmet Acar*, Elinor Ng Eaton*, Kieran T. Mellody*, Christina Scheelb, Ittai Ben-Porathb, Tamer T. Onderbc, Zhigang C. Wangd, Andrea L. Richardson*, Robert A. Weinbergb, c, f, 1, and Akira Orimoa, b, 1

*Cancer Research-UK Stromal-Tumor Interaction Group, Paterson Institute for Cancer Research, The University of Manchester, Manchester M20 4BX, United Kingdom; 1Whitehead Institute for Biomedical Research, Cambridge, MA 02142; 2Department of Biology, Massachusetts Institute of Technology, Cambridge, MA 02139; 3Department of Cancer Biology, Dana-Farber Cancer Institute, Cambridge, MA 02139; 4Department of Pathology, Brigham and Women’s Hospital, Harvard Medical School, Cambridge, MA 02139; and 5MIT Ludwig Center for Molecular Oncology, Cambridge, MA 02139

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Much interest is currently focused on the emerging role of tumor-stroma interactions essential for supporting tumor progression. Carcinoma-associated fibroblasts (CAFs), frequently present in the stroma of human breast carcinomas, include a large number of myofibroblasts, a hallmark of activated fibroblasts. These fibroblasts have an ability to substantially promote tumorogenesis. However, the precise cellular origins of CAFs and the molecular mechanisms by which these cells evolve into tumor-promoting myofibroblasts remain unclear. Using a coimplantation breast tumor xenograft model, we show that resident human mammary fibroblasts progressively convert into CAF myofibroblasts during the course of tumor progression. These cells increasingly acquire two autocrine signaling loops, mediated by TGF-β and SDF-1 cytokines, which both act in autostimulatory and cross-communicating fashions. These autocrine-signaling loops initiate and maintain the differentiation of fibroblasts into myofibroblasts and the concurrent tumor-promoting phenotype. Collectively, these findings indicate that the establishment of the self-sustaining TGF-β and SDF-1 autocrine signaling gives rise to tumor-promoting CAF myofibroblasts during tumor progression. This autocrine-signaling mechanism may prove to be an attractive therapeutic target to block the evolution of tumor-promoting CAFs.

CXCRI4 | Smad | tumor microenvironment | alpha-smooth muscle actin

Myofibroblasts are often observed in the stroma of various human carcinomas that include those of the breast (1). The presence of these cells in large numbers is also associated with higher-grade malignancy and poor prognosis in patients (2–4). Myofibroblasts express α-smooth muscle actin (α-SMA) that distinguishes these cells from fibroblasts and represents a hallmark of activated fibroblasts (5–10). The activated myofibroblast state of stromal fibroblasts also correlates with their ability to promote tumor growth (11–14). Although different types of mesenchymal cells and epithelial cells are proposed to be precursors of the myofibroblasts present in tumors (15–20), their precise cellular origins and functional contributions to tumor growth still remain unclear.

In recent years, the tumor-promoting roles of stromal fibroblasts and α-SMA-positive myofibroblasts, collectively termed carcinoma-associated fibroblasts (CAFs), have been studied (21). CAFs, when inoculated with carcinoma cells, have potently promoted the in vivo proliferation of carcinoma cells and tumor growth in mouse xenograft models (14, 21–25). We previously demonstrated that CAFs, prepared directly from invasive human mammary carcinomas, contain substantial numbers of myofibroblasts that secrete elevated levels of the proangiogenic chemokine, stromal cell-derived factor-1 (SDF-1, also called CXCL12) (14). SDF-1 signaling via its cognate receptor CXCR4, expressed on the surface of carcinoma cells, directly boosts the proliferation of these cells and can stimulate neoangiogenesis by recruiting circulating endothelial progenitor cells (EPCs) into the tumor stroma (14, 26).

Previous research has shed little light on the molecular mechanisms that mediate formation of the myofibroblastic state and the associated tumor-promoting capability of CAFs. The similarities between tumor-associated myofibroblasts and those present in sites of wound healing and chronic fibrosis have long been recognized (5, 27, 28). Though TGF-β-Smad autocrine signaling is apparently responsible for the activated state of myofibroblasts in fibrosis (29–31), it is not known if myofibroblastic CAFs also depend on TGF-β autocrine signaling and/or additional signaling to maintain their unique phenotypes.

We therefore investigated the biochemical alteration(s) underlying the tumor-promoting myofibroblastic phenotype of CAFs and the cellular origins of these cells. Our findings show that the establishment of two autocrine signaling loops, mediated by TGF-β and SDF-1 cytokines, endows resident fibroblasts with the tumor-promoting myofibroblastic phenotype, thereby driving their differentiation into CAF myofibroblasts.

Results

Experimental Generation of CAFs from Human Mammary Fibroblasts. Myofibroblasts have been generated in vitro from fibroblasts through their transdifferentiation following exposure to TGF-β (32, 33). Moreover, the CAF populations in tumor-associated stroma are known to include both fibroblasts and myofibroblasts (14, 34). For these reasons, we speculated that preexisting normal stromal fibroblasts could potentially convert into myofibroblasts in vivo, specifically during the course of tumor progression. Such conversion has not previously been demonstrated.

To test this hypothesis, primary normal human mammary fibroblasts were isolated from reduction mammoplasty tissue and immortalized with hTERT, the catalytic subunit of the telomerase enzyme (35). Retroviral constructs encoding GFP and puromycin-resistance protein were also introduced into these immortalized stromal fibroblasts. The resulting fibroblasts were then mixed with MCF-7 human breast carcinoma cells expressing an activated ras oncogene (36), and the mixtures were injected s.c. into immunodeficient nude mice.

As described in Fig. 1A, the tumor xenografts were resected at 42, 70, and 200 d after implantation and dissociated into single-cell suspensions. These cells were then cultured in vitro in the


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1To whom correspondence may be addressed. E-mail: weinberg@wit.mit.edu or aorimo@piti.man.ac.uk.

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admixed with various
at the indicated days and microvascular density (\( \alpha \)-SMA) was quantified in exp-CAF2 cells relative to the control breast carcinoma cells were injected alone (Fig. 1B), providing additional evidence to support for the myofibroblastic state of these cells.

Western blot analysis further confirmed progressive up-regulation of \( \alpha \)-SMA expression in 42- (5.6-fold), 70- (27.2-fold), 200- (29.1-fold) day-old exp-CAF1 cells, and exp-CAF2 cells (29.8-fold) relative to the control fibroblast-2 cells (Fig. 1C). Exp-CAF2 cells also retained their increased \( \alpha \)-SMA expression, when propagated as pure populations for periods of 14 population doublings (PDs) in vitro following their extraction from tumors (Fig. 1D). Moreover, exp-CAF2 cell populations extracted from four different tumor xenografts also showed similar increased levels of \( \alpha \)-SMA protein expression (Fig. S1C).

We wished to determine whether the myofibroblast differentiation was due to the transfer of the ras oncogene from the admixed MCF-7-ras cells. We therefore checked for the presence of oncogenic ras in the exp-CAFs by PCR and Western blot analyses. No oncogenic ras gene and protein expression could be detected in these cells (Fig. S1D). Collectively, these observations indicate that myofibroblast differentiation is progressively increased during tumor progression and, once established, the differentiated state of the resulting cell populations is stably maintained in a cell-autonomous manner.

To determine the ability of exp-CAFs to promote tumor growth, we performed a tumor xenograft assay. In accordance with previous observations (14), tumors from MCF-7-ras cells admixed with 42-d-old exp-CAF1 or exp-CAF2 cells showed increased growth kinetics and larger volumes by 1.4- or 2.2-fold, respectively, at 147 d after injection compared with tumors containing control fibroblast-2 cells (Fig. 1E, i). Moreover, significant numbers of GFP-positive exp-CAF2 and control fibroblast-2 cells were still present in 150-d-old tumors, as determined by immunofluorescence (Fig. S1E, e and f). Tumors containing 42-d-old exp-CAF1 and exp-CAF2 cells also showed an increase in microvessel density by 2.2- and 5.5-fold, respectively, compared with the control fibroblast-containing tumors (Fig. 1E, ii and Fig. S1E). Taken together, these various observations demonstrate that exp-CAFs closely mimic the tumor-promoting behavior of CAFs extracted from actual human invasive breast carcinomas (14).

**Role of TGF-\( \beta \) Autocrine Signaling Responsible for Myofibroblast Differentiation in exp-CAFs.** As TGF-\( \beta \) autocrine signaling regulates myofibrogenesis during fibrosis, we wondered if this was also the case for the myofibroblast differentiation observed in CAFs. We therefore measured TGF-\( \beta \) mRNA expression in these cells by real-time PCR analysis. Levels of TGF-\( \beta \)1 and -\( \beta \)2 expression progressively increased in 42- (1.6- and 1.6-fold, respectively), 70- (2.0- and 2.2-fold), and 200- (2.4- and 2.4-fold) day-old exp-CAF1 cells and exp-CAF2 cells (2.7- and 4.4-fold) compared with the

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**Fig. 1.** exp-CAFs mimic the tumor-promoting behavior of CAFs prepared from breast cancer patients. (A) Isolation of exp-CAFs. Normal GFP-labeled, puromycin-resistant, immortalized human mammary fibroblasts were coinjected with MCF-7-ras breast cancer cells s.c. into nude mice. Tumors were dissected at the indicated days and dissociated. The injected human fibroblasts were isolated under puromycin selection in culture and were termed experimentally generated CAF1 (exp-CAF1) cells. These cells, resected 42 d after implantation, were once again dissected, dissociated, and cultured in the presence of puromycin. The isolated puromycin-resistant cells were termed experimentally generated CAF2 (exp-CAF2) cells (242 d old).

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. The fibroblasts that survived at the site of injection for the same period as the exp-CAF-2 cells were isolated in the same way and termed control fibroblast-2 cells (242 d old; Fig. S1A). The mesenchymal nature and human origin of exp-CAFs and the control fibroblasts were confirmed by immunofluorescence analysis (Fig. S1B).

**Conversion of Resident Fibroblasts into Tumor-Promoting CAF Myofibroblasts Within the Tumor.** To determine whether the initially admixed normal human fibroblasts had converted into a myofibroblast-rich population during the course of tumor growth, we performed immunofluorescence using an anti-\( \alpha \)-SMA antibody. We observed that ~48% of the total cell population of 242-d-old exp-CAF2 cells stained positive for \( \alpha \)-SMA, a far greater number than the ~14% of myofibroblasts present in the 42-d-old exp-CAF1 and the ~2.5% present in the 242-d-old control fibroblast-2 cell populations (Fig. 1B). The expression level of the extracellular matrix glycoprotein tenascin-C, another marker of myofibroblasts (11, 37), was also dramatically elevated in exp-CAF2 cells relative to the control fibroblast-2 cells (Fig. 1B), providing additional evidence to support for the myofibroblastic state of these cells.

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control fibroblast-2 cells (Fig. 2A, i). TGF-β3 expression, however, remained unchanged (Fig. S2A). We also observed increased TGF-β bioactivity in culture medium conditioned by 42-d-old exp-CAF1 (2.7-fold) and exp-CAF2 (4.5-fold) cells compared with control fibroblast-2 cells (Fig. 2A, ii).

Given the increases in TGF-β expression and bioactivity observed in exp-CAF myofibroblasts, we speculated that TGF-β ligands may induce Smad signaling via their receptor in an autocrine fashion, thereby contributing to the myofibroblastic state of these cells. Indeed, immunofluorescence using an antibody against Smad2/3 revealed intense nuclear staining in exp-CAF2 cells (Fig. 2B, b) and in normal fibroblasts treated with TGF-β1 (10 ng/mL) for 1 h (Fig. 2B, d). In contrast, the nuclear Smad2/3 staining was rarely observed in control fibroblast-2 cells (Fig. 2B, a). Moreover, exp-CAF2 cells treated with SB431542 (10 μM), an inhibitor of the TGF-β type I receptor (TβRI) kinase, for 24 h significantly reduced the level of nuclear staining (Fig. 2B, c), indicating constitutive induction of Smad signaling via activation of the TβRI in these cells. Western blot analysis further confirmed progressive elevation of phosphorylated Smad2 expression in 42- (2.9-fold), 70- (6.2-fold), and 200- (7.1-fold) day-old exp-CAF1 cells and exp-CAF2 cells (14.3-fold) relative to the control fibroblast-2 cells (Fig. 2C).

Taken together, these observations indicate that exp-CAF-secreted TGF-β activates Smad2/3 signaling in these cells in an autocrine fashion, ostensibly through the TGF-β I/II receptor.

To determine if TGF-β autocrine signaling contributes to maintaining the myofibroblastic state and up-regulation of TGF-β expression in exp-CAFs, we generated shRNAs to suppress significantly either TGF-β type II receptor (TβRII) or Smad4 expression (Fig. S2B). Inhibition of Smad signaling by treatment with the TβRII-shRNA, Smad4-shRNA, or SB431542 (10 μM) for 5 d attenuated expression levels of α-SMA (by 85%, 99%, or 85%, respectively), TGF-β1 (by 68%, 48–74%, or 65%), and TGF-β2 (by 66%, 71%, or 75%) in exp-CAF2 cells, compared with the relevant controls (Figs. 2 D and E). Furthermore, activation of Smad signaling by expressing a constitutively active TGF-β1 construct (38), or by adding recombinant TGF-β1, induced α-SMA and TGF-β expression in normal mammary fibroblasts (Fig. S2C), consistent with previous literature (39). Taken together, these findings suggest that activation of TβR-Smad signaling in exp-CAFs induces and maintains their myofibroblastic differentiation and TGF-β synthesis, thereby generating a positive feedback TGF-β signaling loop.

Role of Self-Stimulating SDF-1-CXCR4 Autocrine Signaling in Myofibroblast Differentiation. Elevated expression levels of SDF-1 have previously been observed in CAF myofibroblast populations in vitro and in vivo (14, 40–42). Consistently, real-time PCR analysis showed progressive up-regulation of SDF-1 expression in the 42- (5.9-fold), 70- (10.3-fold), and 200- (38-fold) day-old exp-CAF1 cells and exp-CAF2 cells (34-fold) relative to the control fibroblast-2 cells (Fig. 3A). An ELISA also showed increased levels of SDF-1 released by 42-d-old exp-CAF1 (2.3-fold) and exp-CAF2 (20-fold) cells (Fig. S3A). Moreover, Western blot and immunofluorescence analyses revealed a fivefold increase in expression of the SDF-1 cognate receptor, CXCR4, in exp-CAF2 cells (~14 PDs) relative to the control fibroblasts (Fig. 3B), consistent with previous literature (43). We therefore speculated that the released SDF-1 may act via CXCR4 upon these cells in an autocrine fashion, thus contributing to their myofibroblastic phenotype.

We examined this possibility using two different shRNA constructs against SDF-1 or CXCR4. The SDF-1 and CXCR4 protein expression levels were significantly inhibited by 72–76% and by 66–78%, respectively, in exp-CAF2 cells compared with the effect of control GFP-shRNA (Fig. S3B and Fig. 3C). Immunoblot and immunofluorescence analyses also demonstrated attenuated levels of α-SMA expression in exp-CAF2 cells expressing SDF-1-shRNA (by 67–77%), CXCR4-shRNA (by 62–75%), or treated with AMD3100, a CXCR4-specific inhibitor (44) for 6 d (Fig. 3C and Fig. S3C). These data suggest that SDF-1-CXCR4 autocrine signaling is required for maintaining the myofibroblastic phenotype of exp-CAF2 cells.

To determine whether triggering the SDF-1-CXCR4 signaling induces the myofibroblastic phenotype, a retroviral construct encoding CXCR4 cDNA (45) was introduced into human mammary fibroblasts (Fig. S3D). We observed that α-SMA expression was increased by ~100-fold in these CXCR4-expressing cells when exposed to SDF-1 (200 ng/mL) for 72 h compared with the control GFP-expressing fibroblasts (Fig. 3D). The ligand-induced activation of CXCR4 could also suffice to induce and continuously maintain elevated levels of SDF-1 expression in exp-CAFs (Fig. S3E). Taken together, these findings suggest that, like the described TGF-β autocrine signaling, the SDF-1 autocrine signaling pathway also operates in a self-stimulating fashion and contributes to the myofibroblastic differentiation in exp-CAFs.

Importantly, MCF-7-ras tumors with admixed exp-CAF2 cells expressing either CXCR4-shRNA exhibited a reduction in tumor volume by 49% or 52%, respectively, and in neoangiogenesis by 45% at 128 d after injection compared with tumors containing...
control GFP-shRNA-expressing cells (Fig. 3E and Fig. S3F, a). In contrast, MCF-7-ras tumors that grew in the presence of CXCR4-expressing fibroblasts exhibited a 56% increase in tumor volume at 116 d after injection relative to tumors containing GFP-expressing fibroblasts (Fig. S3F, b). Collectively, these data suggest that SDF-1-CXCR4 autocrine signaling is responsible for the induction and maintenance of the ability of these mesenchymal cells to accelerate tumor growth in vivo.

**Crosstalk Between TGF-β and SDF-1 Autocrine Signaling Loops in exp-CAFs.** As both the TGF-β and SDF-1 autocrine signaling pathways are required for myofibroblast differentiation of exp-CAFs, we reasoned that these pathways may cross-communicate and activate one another to further boost the myofibroblastic phenotype. Indeed, PCR analysis showed that exposure of mammary fibroblasts to TGF-β1 (10 ng/mL) for 24 h up-regulated SDF-1 (four-fold) and CXCR4 (2.5-fold) expression (Fig. 3F, i and Fig. S4A). Inhibition of TβR-Smad signaling by TβRII- (by 70%) or Smad4- (by 62–70%) shRNA attenuated the induction of SDF-1 expression by TGF-β1 in these cells compared with the control GFP-shRNA (Fig. S4B). SDF-1 mRNA expression was also decreased in exp-CAF2 cells expressing TβRII- or Smad4-shRNA by 67% or 74–83%, respectively (Fig. 3F, ii), indicating that SDF-1 expression is mediated by TβR-Smad signaling in these cells. In contrast, inhibition of Smad signaling by Smad4-shRNA failed to suppress CXCR4 expression induced by TGF-β1 (10 ng/mL) for 24 h in mammary fibroblasts (Fig. S4C). Collectively, these data suggest that TGF-β signaling induces and maintains SDF-1-CXCR4 autocrine signaling by elevating SDF-1 expression through a Smad-dependent pathway and by increasing CXCR4 expression through an Smad-independent pathway in mammary fibroblasts.

We further demonstrated that this TGF-β–induced SDF-1-CXCR4 signaling mediates the myofibroblast formation induced by TGF-β. Inhibition of CXCR4 expression by CXCR4-shRNA in mammary fibroblasts incubated with TGF-β1 (10 ng/mL) for 72 h resulted in attenuated induction of α-SMA expression by 3.4-fold compared with the 10.8-fold induction observed in control GFP-shRNA–expressing cells (Fig. 3F, iii). These data indicate the requirement of CXCR4 signaling for the TGF-β–induced myofibroblastic state. We therefore conclude that TGF-β autocrine signaling boosts myofibroblast differentiation in exp-CAFs, not only directly through TβR-Smad signaling pathway, but also through the subsequently induced SDF-1-CXCR4 autocrine signaling pathway. We also observed that activation of SDF-1-CXCR4 signaling induces and helps to maintain elevation of TGF-β expression in exp-CAFs. Inhibition of CXCR4 expression by CXCR4-shRNA resulted in attenuation of TGF-β1 (by 46–59%) and TGF-β2 (by 47–51%) mRNA expression in exp-CAF2 cells compared with the control GFP-shRNA, indicating some dependence of TGF-β expression on SDF-1-CXCR4 autocrine signaling (Fig. 3G). In contrast, exposure of CXCR4-expressing fibroblasts to recombiant 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. S4D). Taken together, these findings strongly suggest that TGF-β and SDF-1 autocrine signaling loops converge to stimulate each other within exp-CAFs.
conclude that the stromal myofibroblasts present within invasive human mammary carcinomas require both SDF-1 and TGF-β autocrine signaling loops in a self-stimulating and cross-communicating fashion to maintain myofibroblast differentiation.

**Discussion**

CAFs, myofibroblast-rich cell populations, extracted from human carcinomas maintain an ability to promote tumor growth when cocultured with carcinoma cells into immuneodeficient mice (14, 46). However, the molecular mechanisms underlying their tumor-promoting ability are poorly understood. Some have reported the importance of somatic genetic alterations in forming the tumor-promoting stroma, yet their existence remains controversial (47–49). The cellular origins of tumor-associated myofibroblasts and the molecular processes that regulate their myofibroblastic state also remain unclear.

In the present experiments, we show that mammary fibroblasts present within a tumor mass can be substantially converted into tumor-promoting CAFs, presumably through their myofibroblast differentiation. We cannot formally exclude the possibility that a small population of α-SMA-positive cells preexisting in normal mammary fibroblasts served as precursors of the tumor-associated myofibroblasts. This alternative origin is rendered less likely, however, by the fact that such myofibroblastic conversion can be efficiently induced in stromal fibroblasts exposed to media conditioned by carcinoma cells (50, 51).

During the course of tumor progression, preexisting stromal fibroblasts acquire progressively enhanced TGF-β and SDF-1 autocrine signaling loops in a self-sustaining fashion that mediate their myofibroblast differentiation and the associated tumor-promoting capability (Fig. 4 B and C). Such autostimulating signaling may fulfill the prerequisites of an epigenetic mechanism that can stably maintain a cellular phenotype—in the present case the myofibroblast differentiation state. We note, in passing, that positive feedback loops are used, for example, to maintain the undifferentiated state of embryonic stem cells and hematopoietic progenitor cells (52, 53).

We imagine that during the course of tumor progression, the autocrine signaling is initially triggered by TGF-β released in significant quantities by carcinoma cells (54–57). TGF-β can elicit enhanced endogenous TGF-β and SDF-1 production by TjR-Smad signaling and induce CXCR4 expression in stromal fibroblasts, thereby facilitating the generation of two autocrine signaling loops, mediated by TGF-β and SDF-1, acting in a positive feedback manner. Such autocrine signaling loops self-stimulate and cross-communicate with each other to maintain the myofibroblastic phenotype.

We cannot exclude the possibility that genetic alterations, acquired in the initially present normal fibroblasts during their incubation within the tumor, influenced the observed autocrine signaling and myofibroblast differentiation. However, we have focused on two types of CAFs—those prepared from human invasive breast carcinomas and those extracted from experimental xenografted tumors—exhibited a normal karyotype and were, when implanted on their own, nontumorigenic (14). We also indicated that wild-type p53 continues to be expressed in both types of CAFs (Fig. S7), strongly suggesting that alteration in p53 signaling is not responsible for the induction or maintenance of such autocrine signaling in tumor-promoting myofibroblasts.

The present observations show that tumor-promoting CAF myofibroblasts can originate from preexisting stromal fibroblasts by establishing TGF-β and SDF-1 autocrine signaling in a cell autonomous fashion during tumor progression. Pharmacological approaches to target and disrupt such autocrine signaling preventing the formation and maintenance of tumor-promoting myofibroblasts may prove to be a useful antitumor therapeutic strategy in the future.

**Materials and Methods**

Primary stromal fibroblasts were extracted from healthy human breast tissue, as described previously (14). The retroviral pMIG (MSCV-IRES-GFP) vector, expressing both hTERT and GFP, and a Pabbe-puro vector encoding a puromycin resistance gene, were infected into these fibroblasts before coimplantation with breast carcinoma cells into nude mice to generate exp-CAFs. Cell culture, immunoblotting, immunostaining, real-time PCR, flow cytometry, ELISA, virus infection, tumorigenicity assay, and evaluation of angiogenesis are performed according to standard procedures. Details are given in SI Materials and Methods. Antibodies, chemicals, and DNA constructs used are also described in SI Materials and Methods.

**Fig. 4.** TGF-β and SDF-1 autocrine signaling operates in CAFs in invasive human breast carcinomas. (A) Immunohistochemistry of sections prepared from invasive human breast carcinomas using antibodies against SDF-1 (a, brown), CXCR4 (b, brown; e and f, red), pSmad2/3 (c, brown; h and i, red), and α-SMA (d, f, g, and i, green). The sections were also stained with hematoxylin (a–c, pale blue) or DAPI (d–i, blue). Cells staining positive are highlighted by arrows. A group of carcinoma cells is indicated by an asterisk. (Scale bar, 50 μm.) (B) During tumor progression, resident stromal fibroblasts within the tumor increasingly acquire two autocrine signaling loops involving TGF-β and SDF-1 that mediate transdifferentiation into tumor-promoting CAF myofibroblasts. (C) Schematic illustration describing two self-stimulating and cross-communicating signaling loops mediated by TGF-β and SDF-1 in CAF myofibroblasts. CAF-secreted TGF-β and SDF-1 ligands act upon TjR and CXCR4, respectively, in an autocrine fashion. The subsequent activation of TjR-Smad2/3 and CXCR4 signaling pathways drives myofibroblast differentiation and endogenous TGF-β and SDF-1 expression, thereby generating self-stimulating autocrine signaling loops that act in a feed-forward manner. Importantly, the TjR-Smad2/3 signaling also induces SDF-1 expression, thereby boosting SDF-1-CXCR4 autocrine signaling. This in turn elevates endogenous TGF-β expression. Cross-talk between these autocrine signaling loops therefore stimulates one another and further boosts myofibroblast differentiation in CAFs. A thick straight arrow indicates direct stimulatory modification, and thin straight arrows depict transcriptional contribution.
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Supporting Information

Kojima et al. 10.1073/pnas.1013805107

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, TjR-II, 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% calf 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 × 10^6) and human mammary fibroblasts (3 × 10^6) were admixed and suspended in 400 μL of medium with 50% Matrigel (BD Biosciences). The mixture was injected s.c. into immunodeficient nude mice. Tumorigenicity 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 × 10^6 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 × 10^5 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.

Immunostaining of Human Breast Tissues and Cultured Mammary Fibroblasts. Mammary fibroblasts were stained using various antibodies as listed 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.
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FCM, flow cytometry; IF, immunofluorescence; IHC, immunohistochemistry; WB, Western blotting.

*Institut Pasteur, Paris.
†Laboratory of Recombinant Therapeutic Proteins, CBA - Advanced Biotechnology Centre, Genoa, Italy.

**shRNA target sequences**

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**PCR primers**

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Kojima et al. www.pnas.org/cgi/content/short/1013805107 2 of 9
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-RasArg-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-β-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-β-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 coincubated 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 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.