Supplemental Data

Stromal Fibroblasts Present in Invasive Human Breast Carcinomas Promote Tumor Growth and Angiogenesis through Elevated SDF-1/CXCL12 Secretion

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Supplemental Experimental Procedures

Isolation of Human Breast Fibroblasts and Cell Culture

Breast tissues were obtained from six independent cases of sporadic invasive ductal breast carcinomas (TNM stage II, SBR grade II-III). Fibroblasts were isolated from cancer- and non-cancer-associated regions of whole breast tissues dissected from whole breast mastectomies as determined by gross examination at the time of surgical excision and subsequent histological analysis. The cancer-associated regions were selected to be minimally necrotic regions of the tumor mass. Non-cancer associated stroma, which was isolated from tissue at least 2 cm. distal to the outer margin of the cancer mass, exhibited normal epithelial and stromal breast histology. Normal fibroblasts were extracted from the breast stroma of a sample obtained from a reduction mammoplasty. Tissues were digested with collagenase type I (1 mg/ml; Boehringer Mannheim) and hyaluronidase (125 units/ml; Sigma) at 37°C with agitation for 12-18 hrs in Dulbecco's Modified Eagle's Medium (DMEM) with 10% fetal calf serum (FCS). The dissociated tissues were incubated without shaking for 5 min at room temperature, followed by the separation of stromal cell-enriched supernatant to a new tube. The stromal fraction was centrifuged at 250 X g for 5 min and the pellet was resuspended in DMEM with 10% FCS and the cells were cultured on tissue culture plates. These primary cultured fibroblasts were isolated in the same way, by tissue dissociation followed by differential sedimentation, plating, and growth in high serum media conditions which select for fibroblast growth. Each fibroblast was then expanded into two 15 cm petri dishes and stored as cells passaged for 2-3 population doublings (PDs) within total 8-10 days after tissue dissociation. We used fibroblasts passaged for up to 5 PDs for subsequent experiments, in order to minimize clonal selection and culture stress which could occur during extended tissue culture.

Data Analysis

Mixed effects models (Pinheiro and Bates, 2000) were used to conduct inference on different aspects of the relationship between cell types, growth rate of tumors formed by MCF-7-ras cells co-mingled various fibroblasts and activated property of fibroblasts measured by collagen gel contraction.

Evaluation of Tumor Growth Rate (mm^3/day)

The basic model for the relationship between tumor volume y and cell type j (CAF, counterpart fibroblast or normal fibroblast) in donor i ($i = 1, \ldots, 6$) is

$$y_{ijklm} = \mu_{i(j)} + \beta_{i(j)} t_m + \gamma_{i(j)} x_{ij} + \delta_{i(j)} x_{ij} t_m + f_{ijkl}(t_m) + e_{ijklm}$$

where y_{ijklm} is the mth volume measurement on tumor l from donor i, cell type j, mouse k, obtained at time t_m.

Parameter $\mu_{i(j)}$ is the marginal mean tumor volume averaged over all observations for which CAF for donor i was compared to cell type j for donor i, $\beta_{i(j)}$ is the average growth in volume per unit time for tumors with type j for donor i, $\gamma_{i(j)}$ is the average difference in tumor volumes between CAF and cell type j, and $\delta_{i(j)}$ is the difference in growth rates between CAF and cell type j. The error term e_{ijklm} has mean zero and, given the values of the random effects to be described, are mutually independent with a constant variance. The random effects introduced by repeatedly measuring multiple tumors in different mice are captured in the (linear) random functions $f_{ijkl}(t_m)$, which are random tumor-specific growth curves specified by parameters that are subject to conventional constraints (conditionally independent mean zero Gaussian, with separate variances to be estimated by restricted maximum likelihood).

The test of $H_0 : \delta_{i(j)} = 0$ is the test for common growth rates in donor i for CAF and type j cells.

Evaluation of Activated Property of CAFs

Activated property of CAFs were measured by collagen gel contraction. Repeated measurement of area (mm^2) of contracted collagen gels containing CAFs or counterpart fibroblasts were modeled as follows:

$$d_{ijkl} = \mu_{ij} + a_k + e_{ijkl}$$

where d is the area of the treated collagen gels, i indexes donor ($i = 1, \ldots, 6$), j indexes cell type (1 = CAF, 2= counterpart), l indexes replicates within experiments, and k indexes experiment (a pair of experiments was performed for donor 1; all other donors provided a single experiment). The parameters μ_{ij} are the type-specific mean areas. Again e_{ijkl} is a measurement error term with mean zero. The ratio of activated property of CAF (evaluated by areas of contracted collagen gels calculated relative to measurements of their cognate counterpart fibroblast) for donor *i* is $\hat{r}_i = \hat{\mu}_{i1}/\hat{\mu}_{i2}$. Small values of $\hat{r}_i$ therefore indicate significant activated property of CAF relative to counterpart.

Correlation between Activated Property of CAFs and the Tumor-Enhancing Ability

The relationship between CAFs' activated property and their tumor-enhancing ability modeled using measurements of tumor volumes of MCF-7-ras tumors containing CAFs. The model used was

$$y_{ijkl} = \mu + \beta t_l + \gamma \hat{r}_i + \delta \hat{r}_i t_l + a_i + b_{ij} + c_{ijk} + e_{ijkl}$$

with i indexing donor, j indexing mouse, k indexing tumor, and l indexing repeated measurements on tumors. Here y denotes tumor volume, μ is overall average tumor volume, and e_{ijkl} is an error term. Random effects a_i are donor-specific departures from the overall mean, b_{ij} are mouse-specific effects nested within donor, and c_{ijk} are tumor-specific effects nested within mouse. All random effects are conditionally independent mean zero Gaussian with separate variance parameters. Parameter δ measures the effect between activated property of CAFs and growth rate (mm^3/day) of tumors formed by MCF-7-ras cells co-injected with CAFs. The test of $H_0 : \delta = 0$ is the test for no effect between them.

All models are fit using restricted maximum likelihood (Pinheiro and Bates, 2000).

Supplemental References

Pinheiro, J., and Bates, D. (2000). Mixed Effects Models in S and S-Plus (New York: Springer).

Supplementary Fig. 1

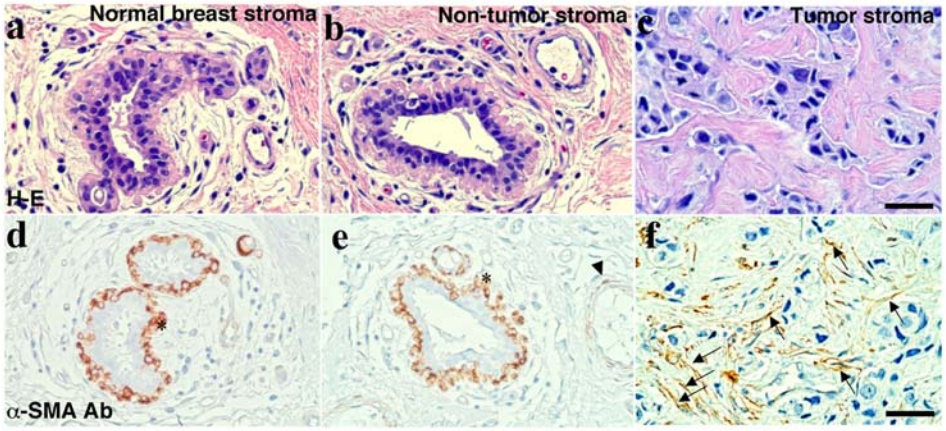

Figure S1. Carcinoma-Associated Stroma Predominantly Produces α-Smooth Muscle Actin (α-SMA)

Parental human breast tissues from which CAFs, counterpart, and normal fibroblasts have been extracted were immunostained by an anti-α-SMA antibody (d, e, f) and also stained with H&E (a, b, c).

The tumor region (c, f) and non-tumor region (b, e) dissected from the breast tissue of the human breast cancer patient, and the normal breast region (a, d) isolated from the healthy patient are shown.

Myofibroblasts (indicated by an arrow) in the tumor region (f) and myoepithelial cells (indicated by asterisks) in non-tumor (e) and normal breast (d) regions are strongly positive for α-SMA. A representative weak signal in pericytes surrounding vasculature is also indicated by an arrowhead (e). Scale bar, 75 μm.

Supplementary Fig.2

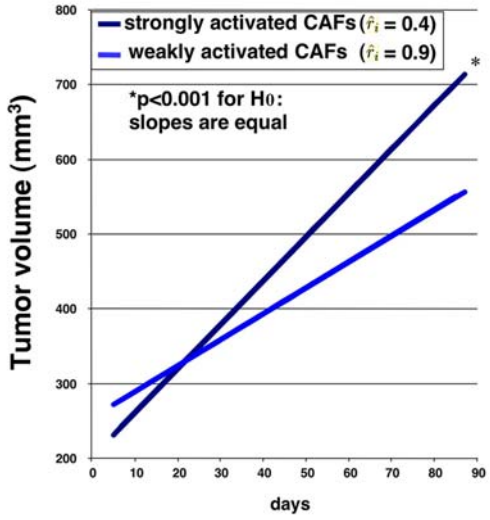

Figure S2. Significant Positive Correlations between Activated Property of CAFs In Vitro and Their Tumor-Enhancing Ability In Vivo

Relationship between activated property of CAFs and their tumor-enhancing ability was analyzed among 6 independent CAFs using mixed effects models (See Supplemental Experimental Procedures). Activated properties of CAFs were evaluated by areas (mm^2) of contracted collagen gels and the relative ratio $\hat{r}_i$ was calculated by comparison to those of their cognate counterpart fibroblasts. In short, smaller values of $\hat{r}_i$ indicate significant CAFs' activated property. And a tumor-enhancing ability was estimated by tumor growth rate (mm^3/day) of tumors formed by MCF-7-ras cells that developed in the presence of CAFs. As the results, strongly activated CAFs ($\hat{r}_i$ = 0.4) show more increased tumor growth rate than do weakly activated CAFs ($\hat{r}_i$ = 0.9) *: $p<0.001$.

Supplementary Fig. 3

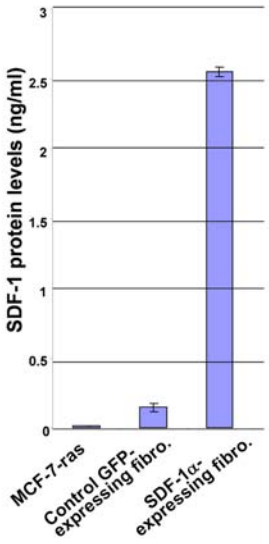

Figure S3. Generation of SDF-1-Expressing Human Breast Fibroblasts

Human SDF-1α or control GFP gene expression vectors were introduced to normal human breast fibroblasts. SDF-1 protein levels in the media conditioned by each fibroblast or MCF-7-ras cells were examined by a SDF-1 ELISA assay. Error bars represent the standard errors of the mean in three independent experiments.