Additional Methods

Cell culture

Cell lines were obtained as follows: A549 human lung carcinoma cells, HCT-116 human colon carcinoma cells, and TOV-112D human ovarian carcinoma cells from ATCC (Rockville, Maryland); MDA-MB-435-β4 human mammary carcinoma cells and GTL16 human gastric carcinoma cells, as previously described (1, 2); MDA-MB-435 human mammary carcinoma cells, from the Georgetown University Tissue Culture Shared Resource (Washington, District of Columbia). It should be mentioned that recent studies suggest a melanoma rather than breast origin for MDA-MB-435 cells (3). Cells were maintained in DMEM (MDA-MB-435, MDA-MB-435-β4) or RPMI (A549, HCT-116, GTL16) supplemented with 10% FBS (Sigma, St. Louis, Missouri). TOV-112D were cultured using a 1:1 mixture of MCDB 105 Medium and Medium 199 plus 15% FBS (all from Sigma).

Pro-SF conversion assays

Factor purification was performed by metal chelate affinity chromatography as previously described (4) from conditioned medium of lentiviral-vector transduced MDA-MB-435 cells. Purified factors at a concentration of approximately 100 ng/μl were stable at 4°C for at least 2 months in the absence of carrier protein. To test factor processability, a fixed amount (50 ng) of pro-SF or uncleavable SF was incubated at 37° C with increasing concentrations of uPA (0-3 μM; Chemicon International, Temecula, California) or fetal bovine serum (0-8%; Sigma, St. Louis, Missouri) in a volume of 50 μl. After 24 hours, proteins were resolved by SDS-PAGE, and the amount of active factor was determined by Western blotting with antibodies against the β-chain of SF (5). For inhibition of pro-SF conversion, 50 ng of Myc-tagged pro-SF were incubated as above with increasing concentrations of uncleavable SF (0-355.55 nM) and either 0.3 μM uPA or 2% FBS. The amount of activated factor was determined by immunoblotting with anti-Myc monoclonal antibodies (SC-40; Santa Cruz Biotech, Santa Cruz, California).

Enzyme-linked immunosorbant assays

Binding of active or unprocessed SF to purified Met was performed by ELISA as described (6). Binding data were analyzed and fit using Prism software (Graph Pad Software, San Diego, California). Quantification of wild-type or uncleavable SF in conditioned medium or plasma was performed by

sandwich ELISA using a monoclonal anti-SF antibody for capture (MAB694, 0.25 μg/well; R&D Systems, Minneapolis, Minnesota) and biotinylated anti-SF antibodies for detection (BAF294, 0.2 μg/well; R&D).

Binding and cross-linking analysis

Displacement of [^{125}I]-labeled uncleavable SF was performed as described (4) using a fixed concentration of [^{125}I]-labeled uncleavable SF (0.25 nM) plus increasing concentrations (0-2,000 nM) of unlabelled uncleavable SF, pro-SF, or active SF. Binding data were analyzed and fit as for ELISA analysis. Cross-linking analysis was performed as described (7) using 1 nM [^{125}I]-labeled uncleavable SF alone or together with 10 μg/ml exogenous uPA (Chemicon) where applicable. Cross-linked proteins were immunoprecipitated using either monoclonal anti-Met antibodies (DQ-13; ref. 8) or monoclonal anti-uPA antibodies (a kind gift of Dr. Blasi, H.S. Raffaele, Milan, Italy), resolved by SDS-PAGE on a 3-10% acrylamide gel, and revealed by autoradiography. Iodinated uncleavable SF was competed with a 1:100 molar excess of cold uncleavable SF, pro-SF, or active SF.

Met activation experiments

For Met phosphorylation experiments, A549 cells were deprived of serum for 72 hours, and then stimulated for 10 minutes with increasing concentrations (0-5.33 nM) of active, wild-type SF or uncleavable SF. Cells were lysed in EB buffer and immunoprecipitated with DQ13 anti-Met antibodies (see above) as described (9). Immunoprecipitated proteins were resolved by SDS-PAGE and analyzed by Western blotting using anti-phosphotyrosine antibodies (UBI, Lake Placid, New York). The same blots were stripped and reprobed with anti-Met antibodies (C-12; Santa Cruz) to normalize the amount of immunoprecipitated receptor. Signal was detected using ECL system (Amersham Biosciences, Uppsala, Sweden) according to the manufacturer's instructions. For inhibition of Met phosphorylation, A549 cells were stimulated as above with a fixed concentration of activated SF (0.44 nM) plus increasing concentrations of pro-SF or uncleavable SF (0-43.40 nM). Met phosphorylation was determined as described above.

RNA analysis

Total RNA was isolated from cells and tumors with RNAwiz (Ambion, Austin, Texas) and retro-transcribed using MMLV Reverse Transcriptase (Promega, Madison, Wisconsin). For amplification of human *met* and of human *app* we used the following primers:

met FW: 5’ AGTGCAGCATGTAGTGATTG 3’

met RV: 5’ TTACGTCAGGATAAGGTGGG 3’

app FW: 5’ CACAGAGAGAACCACCAGCA 3’

app RV: 5’ ACATCCGCCGTAAAAGAATG 3’

In vitro biochemical and biological assays

Analysis of SF-induced Met phosphorylation in lentiviral-vector transduced cells was performed as for untransduced cells, with the only difference that conditioned medium was not removed prior to SF stimulation. For assessment of proliferation rate, lentiviral vector-transduced MDA-MB-435 cells were seeded in 35 mm-dishes ($2 \cdot 10^4$ cells/well) and then cultured in the presence of 4% FBS. Every 48 hours, cells were counted and medium was changed in the remaining plates. For scatter assays, lentiviral-vector transduced A549 cells were seeded in 24-well plates (15,000 cells/well), cultured in the presence of 2% FBS for 3 days, and then stimulated with progressive 1:2 dilutions of recombinant, activated SF (R&D), without removing the conditioned medium (stimulation range: 0-64 ng/ml SF). After 24 hours, cells were fixed with 11% glutaraldehyde, stained with crystal violet, and photographed. The minimal concentration at which scattering was observed was defined scatter threshold (ST). Mitogenic assays were performed in collagen-coated 24-well plates with lentiviral vector-transduced A549 cells ($4 \cdot 10^4$/well) cultured for three days in serum-free medium. Cells were stimulated with recombinant SF (80 ng/ml; R&D) for 12 hours and then pulsed with [^{3}H]-thymidine (1.5 μCi/well; Amersham Biosciences, Uppsala, Sweden) for additional 6 hours. For survival assays, lentiviral-vector transduced MDA-MB-435 cells were cultured in 96-well plates ($5 \cdot 10^3$ cells/well) for 48 hours in 2% FBS either in the absence or presence of SF (80 ng/ml), and then incubated with staurosporine (30 ng/ml; Sigma) for additional 48 hours. Apoptosis was determined by the free nucleosome method (Cell Death Detection ELISA^{PLUS}, Boehringer, Mannheim, Germany). Matrigel invasion assays were performed in 24-well Transwell chambers as described (10) using lentiviral vector-transduced MDA-MB-435 cells in the lower chamber as paracrine uncleavable SF secretors and MDA-MB-435-β4 cells in the upper chamber. Collagen invasion assays were performed using pre-formed spheroids as described (11), and the percentage of sprouted spheroids was scored by microscopy (10).

Experimental metastasis analysis

For experimental metastasis analysis, HCT-116 cells were first transduced with a GFP lentiviral vector (12), and then with the full panel of lentiviral vectors used in this study (empty, wild-type SF, uncleavable SF). Double-transduced cells were injected i.v. into immunodeficient *nu*$^{-/-}$ female mice ($2 \cdot 10^6$ cells/mouse, 6 mice/group), and lungs were extracted for analysis one month later. For each animal, one lung was processed for genomic DNA extraction using a Blood & Cell Culture DNA Kit (Qiagen, Valencia, California), and the second was embedded in paraffin for immunohistochemistry. For TaqMan analysis, experimental lung genomic DNA was amplified using an ABI Prism 7700 instrument (Applied Biosystems, Foster City, California) according to the protocol suggested by the manufacturer. The following oligonucleotides were used as primers: 5'AGCAAAGACCCCAA CGAGAA 3' (GFP forward); 5' GGCGGCGGTCACGAA 3' (GFP reverse). The probe had the following sequence: 5' CGCGATCACATGGTCCTGCTGG 3'. For immunohistochemical analysis, tumor sections were unmasked for 30 minutes at 98° C in citrate buffer (BioGenex, San Ramon, California), and then probed with rabbit polyclonal anti-GFP antibodies (Molecular Probes, Eugene, Oregon).

Tissue analysis

Tumor proliferation was determined using a monoclonal anti-Ki67 antibody as previously described (MIB1; 13). Apoptosis was evaluated with a TUNEL reaction using an In Situ Cell Death Detection Kit (Roche, Basel, Switzerland). Immunofluorescence analysis of tumor vessels was performed on frozen sections using anti-PECAM-1 (CD31 endothelial marker) rat monoclonal antibody (Pharmingen, San Diego, California). Transgene expression and activation of endogenous SF in locally transduced tumors was determined on EB protein lysates (8). Equal amounts of proteins (1 mg) were immunoprecipitated with agarose-conjugated anti-SF antibodies (C-20; Santa Cruz), which immunoprecipitates both human and murine SF, and resolved by SDS-PAGE. For analysis of transgene expression, immunoprecipitated proteins were analyzed by Western blot using anti-human SF antibodies (BAF294; R&D). For analysis of endogenous SF, the same membranes were probed with C-20 antibodies, which can detect only mouse SF in Western blotting. Protein lysates were normalized by Western blot using anti-actin antibodies (C-11; Santa Cruz). Met phosphorylation was determined as above using tumor EB lysates containing 1mM $NaVO_4$ (Sigma).

Analysis of clinical parameters

Bone marrow cells were cytospinned and May Grünwald-Giemsa stained. Liver and kidney sections were stained with hematoxylin and eosin. Both preparations were subjected to histological evaluation by an independent anatomopathologist not informed of sample identity. Bone marrow apoptotic index was calculated on at least 1000 nuclei per mouse on DAPI-stained slides (0.5 μg/ml). Heparin was used for hemochrome determination; all other clinical parameters were measured using citrate, except for BUN and creatine, which were measured in serum. Blood cell count was performed using a Cell-Dyn 3500 automatic analyzer (Abbott Laboratories, Abbot Park, Illinois); coagulation parameters were determined by a Sta-Compact Coagulometer (Junior Instruments, Gennevilliers, France); enzymatic values were measured using a Modular-P800 automatic analyzer (Hitachi, Tokyo, Japan). Wound healing time was measured on a 5-mm wide trans-epithelial lesion generated with a punch-biopsy circular scalpel on the mouse left posterior flank.

Statistical analysis

Statistical significance was determined using a two-tail homoscedastic Student's t-Test (array 1, control group; array 2, experimental group; n = 3-10 depending on the experiment). For all data analyzed, a significance threshold of $p < 0.05$ was assumed. In all figures, values are expressed as mean ± standard deviation, and statistical significance is indicated by a single ($p < 0.05$) or double ($p < 0.01$) asterisk. Power calculation was performed as described (14) using the PS software (http://www.mc.vanderbilt.edu/prevmed/ps/). For analysis of clinical parameters, the detectable alternative was calculated assuming a type I error probability of 0.05, a power of 0.80, n = 5, and the appropriate σ value. For all figures, the data generated in vitro are representative of at least two distinct experiments conducted in triplicate. In the case of in vivo data, the number of mice employed varies and is indicated for each experiment.

References to Additional Methods

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