Supporting Information

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SI Materials and Methods

Cell Culture. MCF-7 cells were maintained in RPMI with 10% FBS, 5 μg/mL insulin, and antibiotic/antimycotic. SUM159 cells were maintained in Ham's F-12 medium with 5% FBS, 5 μg/mL insulin, 1 μg/mL hydrocortisone, and antibiotic/antimycotic. MDA-MB-231 cells were maintained in RPMI with 5% FBS and antibiotic/antimycotic. Hypoxic conditions were achieved using a modular incubator chamber (Billups-Rothenberg) containing 1% O_2, 5% CO_2, and balance N_2. Oxygen levels were monitored with an O_2 detector (Sperian).

Drug Treatment and Dissociation of Tumors. Mice were treated daily with sunitinib or vehicle control by oral gavage. Bevacizumab was injected intraperitoneally twice a week. Tumors were measured by digital calipers twice weekly. At the end of drug treatment, the mice were humanely euthanized and tumors were harvested. Tumor tissues were dissociated mechanically and enzymatically to obtain a single-cell suspension. Tumors were minced by scalpel and incubated in Medium 199 (Invitrogen) mixed with collagenase/hyaluronidase (STEMCELL Technologies) at 37 °C for 60 min. The tissues were further dissociated by pipette trituration and then passed through a 40-μm nylon mesh to produce a single-cell suspension, which was used for Aldefluor assay and flow cytometry. Before Aldefluor assay, tumor cells were stained with mouse anti-mouse H-2Kd antibody (553563) and APC goat anti-mouse IgG secondary antibody (550826), both from BD Pharmingen.

Secondary NOD/SCID Mouse Model. Living cells from the dissociated tumors were sorted out by fluorescence-activated cell sorting. Mouse cells were removed based on H-2kd staining. Two groups of mice were implanted with tumor cells separately. Each secondary non-obese diabetic/severe combined immunodeficient (NOD/SCID) mouse was inoculated with 50,000, 5,000, or 500 cells from either control mouse tumors or sunitinib-treated tumors in one of the inguinal mammary fat pads. The growth of tumors was monitored and tumor sizes were measured weekly.

Immunohistochemistry. Formalin-fixed, paraffin-embedded tissue was sectioned, dewaxed, and rehydrated through graded alcohol. Sections were heated to 98 °C for 40 min in 0.01 M sodium citrate buffer (pH 6.0) for antigen retrieval. For ALDH1-Hypoxyprobe double labeling, sections were blocked for 30 min in 0.1% Nonidet P-40 (Sigma), 50% normal goat serum (NGS) (Invitrogen), and 1% BSA (Sigma) in PBS (pH 7.3). Each step was followed by two washes in PBS. Samples were incubated with rabbit anti-pimonidazole antibody (Hypoxyprobe) diluted in PBS overnight at 4 °C, and then with DyLight goat anti-rabbit 488 (green) antibody (Pierce) diluted in PBS for 30 min at room temperature. This was followed by incubation with mouse anti-ALDH1 (611194) (BD Biosciences) diluted in PBS overnight at 4 °C, and with DyLight goat anti-mouse 546 (red) antibody (Pierce) diluted in PBS for 30 min at room temperature. Coverslips were mounted with ProLong Gold Antifade plus DAPI (Invitrogen). β-Catenin staining was done using the same antigen retrieval as above. Tissue was blocked for 30 min in 0.25% Triton X-100, 50% NGS, and 1% BSA (Sigma) in PBS (pH 7.3). Samples were incubated with mouse Ab2081 from Millipore overnight at 4 °C, and then with goat anti-mouse Alexa Fluor 488 secondary antibody (Invitrogen) for 30 min at room temperature. CD31 staining was carried out with the same antigen retrieval methods as above. Following blocking with BSA and NGS, samples were incubated with rabbit anti-CD31 (sc-1506) (Santa Cruz Biotechnology) overnight at 4 °C and then with goat anti-rabbit Alexa Fluor 488 for 30 min at room temperature. Pictures were taken on a Leica DMIRB fluorescence microscope using a Hamamatsu digital camera and MetaMorph software (Molecular Devices). Immunohistochemical staining of Hypoxyprobe was carried out with a Histostain-Plus Broad Spectrum Kit (Invitrogen) and 3,3'-diaminobenzidine (DAB) (Vector Labs) according to the manufacturer's directions.

Immunoblotting. Primary antibodies used for Western blotting were mouse anti-Akt (2920), rabbit anti–phospho-Akt S473 (9271), mouse anti–phospho-S552-β-catenin (9566) (Cell Signaling Technology), mouse anti–β-catenin (610154) (BD Biosciences), and mouse anti–β-actin (sc-8432) (Santa Cruz Biotechnology). Hypoxia-inducible factor (HIF) 1α antibody (NB100-479) was from Novus Biologicals; HIF-2α antibody (NB100-122) used in Fig. 4 was also from Novus Biologicals. In addition, we used HIF-2α antibody (NB100-132) from Novus Biologicals and HIF-2α antibody (ab199) from Abcam without being able to detect HIF-2α expression in cells. We used up to a 1:50 dilution of the primary antibody with an overnight incubation as well as an overnight exposure of the film without being able to detect the protein. The HIF-2α–positive control used was from Novus Biologicals (NBL1-10286).

Fig. S1. Tumor vascularity was determined by CD31 staining of sections from control and sunitinib- and bevacizumab-treated tumors. The number of blood vessels present in the field of each captured image was counted. Data are shown as averages ± SD. n = 8. *$P < 0.001$.

Fig. S2. (*A*) Live cells derived from the dissociated primary tumors were sorted out by fluorescence-activated cell sorting. Mice were implanted with tumor cells from either control mice or sunitinib-treated mice. Each secondary NOD/SCID mouse was inoculated with 50,000, 5,000, or 500 cells in one of the inguinal mammary fat pads. The growth of tumors was monitored weekly. n = 3–4. *$P \leq 0.05$. (*B*) Cell number and cell viability as determined by trypan blue exclusion are shown for MDA-MB-231 tumor samples from control and sunitinib-treated mice following the final treatment. The percentage of dead cells is given for each tumor.

Fig. S3. Bevacizumab (Avastin) increases the tumor stem/progenitor cell population. (*A*) MDA-MB-231 cells were injected directly into the inguinal fat pads of NOD/SCID mice. When tumors reached an average size of 4 mm in diameter (day 26), mice were administered either vehicle control or bevacizumab (5 mg/kg) twice weekly. Tumor measurements were recorded twice a week. Data are shown as averages ± SD. n = 14–16. *$P < 0.01$. (*B*) Photographs of tumors from vehicle control and bevacizumab-treated mice. Note the lack of vasculature in the bevacizumab-treated tumors. (*C*) The percentage of $ALDH^{+}$ cells was determined by Aldefluor assay in tumors from vehicle control and bevacizumab-treated mice. Data are shown as averages ± SD. n = 8. *$P < 0.001$. (*D*) Cell number and cell viability as determined by trypan blue exclusion are shown for tumor samples from control and bevacizumab-treated mice following the final treatment. The percentage of dead cells is given for each tumor.

Fig. S4. Hypoxyprobe immunohistochemical staining of a sunitinib-treated tumor section exhibiting necrosis. The most intense Hypoxyprobe staining (indicated by arrow) is located directly adjacent to the necrotic zone (N) demonstrating the specificity of the staining for hypoxic areas.

Fig. S5. SUM159 and MDA-MB-231 cells were seeded in 96-well microplates at a density of 5,000 cells per well. Cells were treated with increasing concentrations of sunitinib as indicated. After 48 h of incubation, cell viability was assessed by methylthiazolyldiphenyl-tetrazolium bromide (MTT) assay (Sigma), and the concentration that induced 50% growth inhibition (IC_{50}) was determined to be of ~30 μmol/L for both cell lines. Data are shown as averages ± SD. $n = 3$.

Fig. S6. SUM159 and MDA-MD-231 cells were grown in the presence of 0, 0.1, 0.5, or 1.0 μM sunitinib (SUM159: 2-d treatment; MDA-MB-231: 4-d treatment). The percentage of Aldefluor⁺ cells following treatment was then assessed by the Aldefluor assay. No significant change in the Aldefluor⁺ population was detected. Data are shown as averages ± SD. n = 3.

Fig. S7. ALDH1⁺ and ALDH1⁻ SUM159 cells were sorted by flow cytometry using the Aldefluor assay (STEMCELL Technologies) and grown under 21% or 1% oxygen for 3 d. RNA was extracted from the cells using the RNeasy Mini Kit from Qiagen. Gene expression was analyzed using an RT^2 Profiler PCR Array (SABiosciences). ALDH1A expression was significantly higher in Aldefluor-positive cells. However, no significant change in ALDH1A expression was detected following hypoxia treatment. Data are shown as averages ± SD. n = 3. *P < 0.01.

Fig. S8. SUM159 cells were plated in 10-cm dishes and allowed to attach overnight. Cells were transfected with either control siRNA or siRNA oligos targeting HIF-1α. After 24 h, media were changed and cells were moved to 1% O_2 for a total of 72 h. Cell viability was calculated following trypsinization of cells by trypan blue exclusion. (*A*) No significant differences were found in the percentage of nonviable cells in either transfected or untransfected groups after hypoxia treatment. Data are shown as averages ± SD. (*B*) Hypoxia treatment results in slower cell growth of SUM159 cells. This effect was abrogated by HIF-1α knockdown by siRNA. $n = 12$ (four independent experiments with triplicates). $*P < 0.05$.