Supporting Information

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SI Materials and Methods

Cell Proliferation Assay. DNA was extracted from TC-71 cells by subjecting them to three freeze–thaw cycles in double-distilled water (5 min in liquid nitrogen and 15 min in water at room temperature) followed by 15 min of sonication. Cell lysates, assay buffer, and a dye solution were combined in an opaque 96-well plate and incubated for 10 min at room temperature. Excitation and emission wavelengths of 485 and 528 nm, respectively, were used to measure the fluorescence (FLx800 fluorescence microplate reader; BioTek Instruments). A lambda DNA standard curve was used to measure DNA concentrations. Sample cellularity was obtained by dividing total DNA per sample against DNA content per cell (derived from DNA extracts from known number of TC-71 cells).

Immunohistochemical Analysis. Staining for CD99, pancytokeratin S100 protein, and smooth muscle actin was performed in a Clinical Laboratory Improvement Amendments-approved clinical laboratory using an automated stainer (Dako or Leica Microsystems) following the manufacturer's instructions and commercially available antibodies against CD99 (Dako), a cytokeratin mixture (Dako, BD Biosciences, Invitrogen), smooth muscle actin (Sigma-Aldrich), and S100 protein (Biogenex). Either 3-amino-9-ethylcarbazole or 3,3′-diaminobenzidine (DAB) was used as a chromogen, and sections were counterstained with hematoxylin. Positive and negative controls were run in parallel. For insulin-like growth factor 1 receptor (IGF-1R), phosphorylated IGF-1R (pIGF-1R), and c-kit, sections were washed and subjected to antigen retrieval for 20 min (c-kit and pIGF-1R) or 45 min (IGF-1R). After antigen retrieval, the slides were cooled, washed in PBS/Tween-20 [PBST, 0.05% (vol/vol)], and 0.3% (vol/vol) H_2O_2 was added for 15 min. Slides were then washed in PBST and blocked for 30 min using serum-free protein block. Slides were incubated with c-kit (Epitomics) or pIGF-1R (Abcam) or IGF-1R (Invitrogen) primary antibodies overnight. Thereafter, slides were washed and biotinylated secondary antibodies was added for 30 min, followed by streptavidin for 30 min. The slides were developed using DAB kit (Dako).

Flow Cytometry. Cell surfaces were labeled directly with fluorescein isothiocyanate-CD99, phycoerythrin–IGF-1R, phycoerythrin–Cy5-cKit, and allophycocyanin-human epidermal growth factor receptor 2 monoclonal antibodies. Caspase-3, Ki-67, and cleaved poly(ADP-ribose) polymerase (PARP) were labeled intracellularly after cell permeabilization using a kit obtained from eBioscience and corresponding conjugated monoclonal antibodies. The stained cells were fixed, acquired using a FACSCanto II flow cytometer (BD Biosciences), and analyzed using the FlowJo software program (Tree Star). All antibodies used were obtained from BD Biosciences.

Reverse-Phase Protein Array and Bioinformatics Analysis. Using a 2470 arrayer (Aushon BioSystems), 1,056 sample arrays were created on nitrocellulose-coated FAST slides (Schleicher & Schuell BioScience). Slides were probed with 152 validated primary antibodies, and signals were amplified using a DakoCytomation-catalyzed system (Dako). Secondary antibodies were used as a starting point for amplification. Slides were scanned, analyzed, and quantified using the MicroVigene software program (VigeneTech). This program provides automated spot identification, background correction, and individual spot-intensity determination (expressed in logarithmic units). The resulting data were normalized for possible unequal protein loading, taking into account signal intensity for each sample for all antibodies tested. Log_2 values were median-centered by protein to account for variability in signal intensity by time and using the formula $\log_2$ signal − $\log_2$ median. The data were analyzed using GeneSpring GX software program (version 12.1; Agilent Technologies) by performing one-way ANOVA followed by Tukey's test to compare each pair of group to borrow strength from more samples to estimate the between-group variance with minimum twofold change. We found 25 proteins significantly associated with difference between pair groups by hierarchically clustering the data with Euclidean correlation, Pearson centroid linkage, and Benjamini–Hochberg false discovery rate correction at 0.05.

In Vivo TC-71 Xenograft Tumor Growth. Mice bearing s.c. tumors were administered MK-0646 or MK-8669 (Merck) when their tumors reached a diameter of 6 mm or doxorubicin (Pfizer) when their tumors reached a diameter of 10 mm. All drugs were administered intraperitoneally (i.p.) to mice in groups of four, with IGF-1R inhibitors given twice a week (1 mg per animal), mammalian target of rapamycin (mTOR) inhibitors given five times a week (5 $mg \cdot kg^{-1} \cdot d^{-1}$), and doxorubicin given as a single i.p. injection (12, 10, 6, 3, 1.2, and 0 mg/kg). Tumor volumes (in cubic centimeters) were measured at the initiation of the study and two or three times a week for up to 40 d afterward. Tumor volumes were calculated using the formula $(\pi/6) \times D \times d^2$, in which D is the largest diameter and d is the smallest diameter.

Statistical Analysis. Percentage cell viability is expressed as mean ± SEM. Statistical differences between groups in Fig. 4 were determined by performing a two-factor ANOVA before making multiple pairwise comparisons at each drug concentration using the Tukey's honestly significant difference procedure at a significance level of 95%.

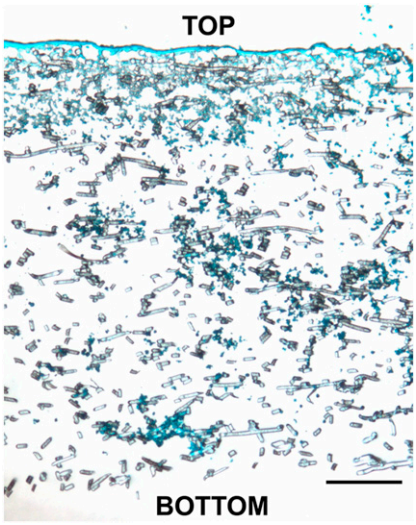

Fig. S1. Fast Green-stained cross-section of the 3D EWS construct after 20 d in culture. Cells were found throughout the scaffold, with the majority located within the top one-fifth of the scaffold. (Scale bar, 200 μm.)

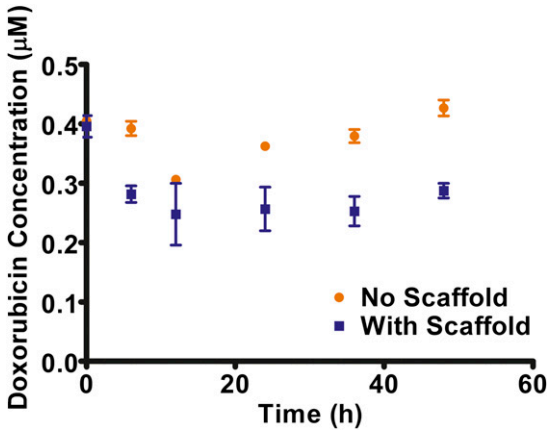

Fig. S2. Adsorption of doxorubicin onto 3D poly(ε-caprolactone) (PCL) scaffolds. The 0.5 μM doxorubicin in serum-containing RPMI medium (Dox/medium) was prepared, and 3 mL was pipetted into individual wells of 12-well plates. PCL scaffolds ($n = 4$) were immersed in Dox/medium and measurements of doxorubicin fluorescence were taken at 0, 6, 12, 24, 36, and 48 h. Wells that contained only Dox/medium (no scaffold) served as controls ($n = 4$). Forty-eight hours was selected as the last time point given that Dox/medium was changed every 2 d in the doxorubicin studies represented by Fig. 4. At each time point, Dox/medium from each sample was collected; excitation and emission wavelengths of 485 and 590 nm, respectively, were used to measure the fluorescence intensity of doxorubicin (SpectraMax M2; Molecular Devices). The concentration of free doxorubicin (not adsorbed) was calculated from a standard curve freshly prepared at each time point. As shown, even though 0.5 μM Dox/medium was prepared, the concentration of free doxorubicin measured at time $t = 0$ is ~0.4 μM, reflecting possible adsorption of the drug onto the polystyrene vessel that was used to contain the Dox/medium before it was pipetted into wells. Statistical analysis by two-way ANOVA indicated that significantly more doxorubicin adsorbs onto the PCL scaffold compared with the tissue culture plastic well alone ($P < 0.05$). However, Fig. 4*A* shows that, within the concentration range of doxorubicin after adsorption onto the PCL scaffold, cytotoxicity in 2D is still greater than 90%, indicating that the reduced drug sensitivity observed in 3D is not due to the decreased availability of doxorubicin after adsorption onto the PCL scaffold.