Figure S4. (a) The cell number declined in cultures of Y40/51/21 tubes. Cells were subjected to a tube assay and at the indicated times, the cells were recovered from the collagen gel, the viable cell number determined by trypan blue exclusion. (b) The amount of cleaved caspase-3 increased in cultures of Y40/51/21 tubes. Same as panel a, expect that the cells recovered from the gels were lysed and the lysate was subjected to Western blot analysis using anti-cleaved caspase-3 and anti-RasGAP antibodies. (c) Apoptosis was readily detected in the cultures that had undergone regression. Same as panel a, except the tubes were fixed, and then frozen in OCT medium. The samples were sectioned and subjected to a TUNEL assay. Green fluorescence staining indicates TUNEL positive cells, and the blue color is DAPI, which marks nuclei. Bar, 50 µm. (d) Changes in actin were detected when tubes regressed into aggregates. Cells were subjected to a tube assay and at the indicated times the tubes were fixed, and then frozen in OCT medium. The samples were sectioned and stained with rhodamine-conjugated phalloidin. Bar, 50 µm.

Supplementary Material and Methods

Antibody. Rabbit polyclonal anti-cleaved caspase-3 antibody was obtained from Cell Signaling Technology (Beverly, MA).

Trypan blue exclusion assay. Cells were plated in a collagen sandwich gel and incubated for the indicated times. The gel was dissolved, the cells were recovered and resuspended in culture media. A 1:1 mixture of cells:trypan blue(0.4 %) was prepared and the number of cells that excluded trypan blue was counted in a hemacytometer.

TUNEL assay. Cells organized into tubes were fixed with 4 % paraformaldehyde for 10 min at room temperature. After PBS washing, tubes in a collagen sandwich gel were embedded in OCT medium and frozen immediately. The OCT-embedded tube blocks were cryosectioned, washed with PBS for 20 min, incubated with proteinase K solution (40 ng/µl) for 30 min at 37 °C, and washed again with PBS for 2 min, 4 times. Sections were subjected to the dUTP nick end labeling TUNEL assay according to the manufacturer’s protocol (Upstate Biotechnology). The biotin-labeled cleavage sites were then visualized by reaction with fluorescein conjugated avidin. Nuclei were identified by labeling for 15 min at room temperature with 500 ng/ml DAPI (Sigma). Sections were washed in PBS and permanently mounted with Vectorshield (Vector laboratories Inc., Burlingame, CA). UV fluorescence images were obtained with a digital camera on a ZEISS axioskop microscope (Carl ZEISS Inc., Göttingen, Germany).

Phalloidin staining. OCT-embedded tube blocks were made as described above. After cryosection, sections were washing with PBS 3 times for 5 min each, permeabilized with 0.2% Triton-X100 in PBS for 5 min, and incubated with rhodamine-phalloidin (6.6 µM, Molecular Probes, Eugene, OR) diluted 1:100 in PBS for 15 min. Sections were washed in PBS and mounted with Vectorshield. Fluorescence images were obtained with a digital camera on a ZEISS axioskop microscope.