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.

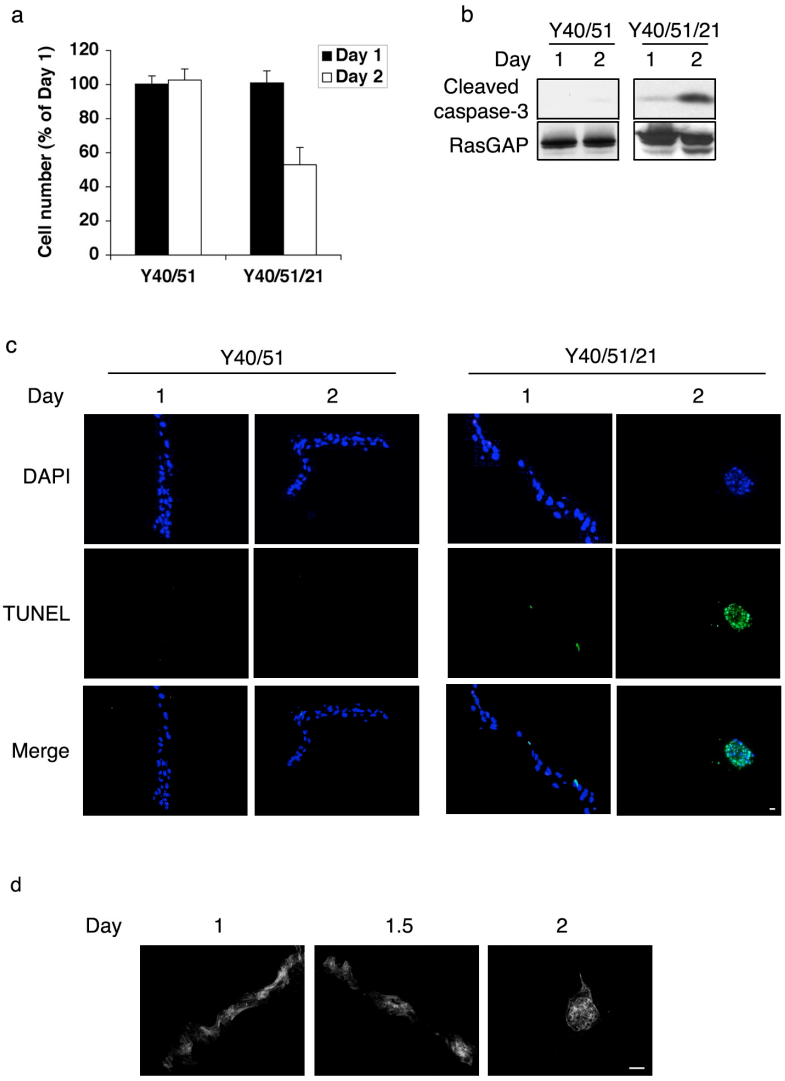
Supplementary Material and Methods

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.


a
Cell number (% of Day 1)
120
100
80
60
40
20
0
Day 1
Day 2
Y40/51
Y40/51/21
b
Y40/51
Y40/51/21
Day
1
2
1
2
Cleaved caspase-3
RasGAP
c
Y40/51
Y40/51/21
Day
1
2
1
2
DAPI
TUNEL
Merge
d
Day
1
1.5
2