Supplemental Information

Supplemental Materials and Methods

Cell culture. HMT-3522 mammary epithelial cells were cultured in H14 medium (DMEM/Ham's F12 containing insulin at 250 ng/ml, transferrin at 10 μg/ml, sodium selenite at 2.6 ng/ml, 1×10^{-10} M estradiol, 1.4×10^{-6} M hydrocortisone, and prolactin at 5 μg/ml) (1, 2). The nonmalignant HMT-3522-S1 cells were propagated on plastic in medium containing 10 ng/ml epidermal growth factor (EGF). Also derived from the same parental line, malignant HMT-3522-T4-2 cells were propagated on collagen type I (PureCol™, Inamed Biomaterials)-coated flasks in the absence of EGF (1, 2). MDA-MB-436, MDA-MB-231, Hs578T, SK-BR-3, MDA-MB-361, MCF7 human breast cancer cell lines were maintained on tissue culture plastic in DMEM/H-21 (Invitrogen) containing 10% fetal bovine serum.

Real-time PCR analysis. The following primers were used to amplify total fibronectin, EDA+ fibronectin and 18S rRNA cDNA sequences. Total fibronectin, 5'- CCAAGCTCAA GTGGTCCTGT-3' (forward primer) and 5'-CACTTCTTGGTGGCCGTACT-3' (reverse primer); EDA+ fibronectin, 5'-CCCTAAAGGACTGGCATTCA-3' (forward primer) and 5'-GTGGACTGGGTTCCAATCAG-3' (reverse primer); 18S rRNA, 5'-TCGGAACTGAGGC CATGATT-3' (forward primer) and 5'-CCTCCGACTTTCGTTCTTGATT-3' (reverse primer). Fragments were amplified with following protocol: 95ºC for 10 min (initial denaturation) and 45 amplification cycles (95ºC for 5 s, 49 ºC for 5 s, 72ºC for 10 s). Fibronectin mRNA was normalized to the corresponding 18S rRNA and averaged from three independent experiments. Melting curve was analyzed to verify the presence of a single PCR product.

Lysis from 3D lrECM. To release cells from 3D lrECM, cultures were first treated with ice-cold PBS containing 5 mM EDTA and then cells were lysed in 1% radioimmunoprecipitation

assay (RIPA) buffer [1% Noidet P-40, 150 mM NaCl, 50 mM Tris-HCl, pH7.4, 5 mM EDTA, 1% Na-deoxychorate, 0.1% SDS, 1 mM Na_3VO_4, 10 μM Na_2MoO_4, and protease inhibitor cocktail (Calbiochem)]. After sonication, the solution was centrifuged and the supernatant is collected (2).

Immunoprecipitation. Cell lysates with adjusted protein concentration were incubated with indicated antibody and ProteinG-Sepharose™4 fast flow (GE Healthcare) for 2 h or overnight at 4ºC, and then washed three times with 1ml ice-cold lysis buffer. The complexes retained on the beads were separated by SDS-PAGE, and then subjected to immunoblotting.

Immunoblotting. Protein was aliquoted onto Novex® 4-20% Tris-Glycine gels (Invitrogen) in equal amounts and separated using low voltage current. Protein bands were transferred onto nitrocellulose membrane (Whatman), and blots were blocked with 5% skim milk/TBST. Blots were probed with primary antibodies (1:1,000 dilution in 3% skim milk/TBST), then washed, incubated with secondary antibodies (1:1,000 dilution in 3% skim milk/TBST), and exposed by enhanced chemiluminescence (Super signal® West Femto Maximum Sensitivity substrate; Thermo scientific). Signals were captured with the FluorChem 8900 imaging system.

Immunostaining. Cells from 3D cultures were fixed onto a slide glass with 4% paraformaldehyde at room temperature for 15 min, washed three times with glycine/PBS for 10 min, and blocked with blocking buffer [10% goat serum (Invitrogen), 1% Goat F (ab') 2 anti-mouse antibody (Invitrogen) in IF wash buffer (0.05% NaN_3, 0.1% BSA, 0.2% Triton-X 100 and 0.05% Tween 20 in PBS)] for 1.5 h at room temperature in moist chamber (3). After removing blocking buffer, samples were incubated with anti-α5-integrin antibody (IIA1) diluted 1:50 in blocking buffer for 2 h at room temperature, and then washed three times with IF wash buffer for 20 min, followed by incubating with AlexaFluor488-conjugated secondary antibody (Invitrogen)

for 40 min at room temperature in dark moist chamber, and then washed three times with IF buffer for 20 min. Nuclei were counterstained with 4', 6-diamino-2-phenylindole (DAPI; Sigma, St. Louis, MO), washed twice with PBS for 10 min, and mounted. Fluorescent images were acquired by a Zeiss LSM410 inverted laser scanning confocal microscope. Images were captured at the mid-section of each 3D-grown colony with same intensity and exposure times. Images were pseudocolored using Adobe Photoshop.

Antibodies. Anti-β1-integrin, clone 18 (BD Biosciences), AIIB2 (Sierra Biosources, Morgan Hill, CA); polyclonal anti-α5-integrin (Millipore); monoclonal anti-α5-integrin, P1D6 (Millipore), IIA1 (BD Biosciences); anti-fibronectin, IST-4 for total fibronectin (Sigma), IST-9 for EDA+ fibronectin (Abcam, Cambridge, MA); anti-Akt (Cell Signaling Technology, Danvers, MA); anti-phospho-Akt (Cell Signaling Technology); anti-β-actin (Sigma); ECL™ anti-mouse IgG, horseradish peroxidase linked whole antibody (from sheep), NA931V (GE Healthcare) ; ECL™ anti-rabbit IgG, horseradish peroxidase linked whole antibody (from donkey), NA934V (GE Healthcare).

Apoptosis assay. Apoptosis was detected using terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) in samples taken from 3D lrECM cell culture. Samples from 3D lrECM cultures were fixed onto glass slides in 4% paraformaldehyde and permeabilized in 0.1% Triton X-100 in 0.1% sodium citrate. After washing, cells were incubated in TUNEL reaction mixture (In Situ Cell Death Detection Kit, Roche) at 37ºC for 60 min and then stained with 4', 6-diamino-2-phenylindole at room temperature for 5min. After washing, the samples were mounted.

Akt kinase assay. Akt kinase activity of 3D lrECM cultured cells was detected using a nonradioactive Akt kinase assay kit (Cell Signaling Technology) as described manufacture's

instruction and previously (4). Briefly, cells from 3D cultures were released from lrECM using ice-cold PBS containing 5 mM EDTA and then cells were lysed in cell lysis buffer. Cell lysates were incubated with 20 µl of immobilized phospho-Akt (Ser473) rabbit antibody beads slurry for overnight at 4ºC, and then washed twice with 500 µl of lysis buffer and 500 µl of kinase buffer. After immunoprecipitation of phospho-Akt, beads were incubated with 1 µl of 10 mM ATP and 1 µl of GSK-3 fusion protein for 30 minutes at 30 °C, and then terminated reaction with SDS sample buffer. The samples were separated by SDS-PAGE, and then subjected to immunoblotting with phospho-GSK-3α/β (Ser21/9) (37F11) rabbit monoclonal antibody.

References

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Supplemental Figure Legends

Supplemental Figure 1. Treatment with ATN-163 did not induce apoptosis in T4-2 cells in 3D lrECM.

A, Experimental schema of the 3D lrECM assay used in this study. T4-2 cells were allowed to form malignant colonies for 4 days prior to 72 hours of treatment with ATN-163.

B, Phase-contrast micrographs of T4-2 cells colonies cultured in 3D lrECM with ATN-163 are shown. Bar, 50 μm.

C, Treatment with ATN-163 did not induce apoptosis of T4-2 cells cultured in 3D lrECM, measured by counting TUNEL-positive cells. *Columns*, mean (n=3); *bars*, SE.

Supplemental Figure 2. αv-integrin expression in S1 and T4-2 cells in 3D lrECM.

Western blot for αv-integrin subunits from total cell lysates shows upregulation of αv-integrin in malignant T4-2 human breast cells compared to non-malignant S1 cells cultured in 3D lrECM. Equal amounts of protein were loaded and subjected to immunoblotting. *Columns*, mean intensity of western blot analysis (n=4); *bars*, SE. ***, $P < 0.001$.

Supplemental Figure 3. Akt phosphorylation was not affected by inhibition of α5β1-integrin and fibronectin interactions in non-malignant S1 cells in 3D lrECM.

S1 cells in 3D lrECM were treated with or without 0.5 mg/ml ATN-161. The level of phosphorylated Akt relative to total Akt is shown, *Columns*, mean (n=3); *bars*, SE. n.s., not significant.