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Supplemental materials and methods

Cultured cells, chemicals, and antibodies

MCF-10A cells were obtained from American Type Culture Collection and cultured as previously described (Debnath et al., 2003). Growth factor–reduced Matrigel™ (BD Biosciences) lots with protein concentrations between 9–11 mg/ml were used. OHT was obtained from Sigma-Aldrich. Rapamycin was originally obtained as a gift from J. Blenis (Harvard Medical School) and subsequently purchased from Calbiochem. For immunofluorescent staining, the following antibodies were used: human specific α-laminin V antibodies from Chemicon; α-Ki-67 from Zymed Laboratories; and α-activated caspase-3, α-phospho-Akt (Ser^{473}, IHC specific), α-phospho-FKHR/FKHR-L1 ($Thr^{24/32}$), α-phospho-ERM ($Thr^{567/564/558}$), and α-phospho-mTOR (Ser^{2448}) from Cell Signaling Technologies. For immunoblot analysis, the following antibodies were used: α-phospho-Akt (Ser^{473}), α-phospho-Akt (Thr^{308}), α-phospho-Akt substrate, α-phospho-TSC2 (Thr^{1462}), α-phospho-p70S6K (Thr^{389}), and α-p70S6K from Cell Signaling Technologies; α-TSC2 (C-20) from Santa Cruz Biotechnology, Inc.; and α-phospho-FKHR-L1 (Thr^{32}), α-FKHR-L1, α-phospho-GSK-3α (Ser^{21}), and α-GSK-3 (4G-1E) from Upstate Biotechnology. α-Akt1 has been previously described. Antibodies for immunoblotting include α-HA (HA-11) antibody (Babco) to detect the ER-Akt fusion, α-cyclin D1 (Santa Cruz Biotechnology, Inc.), and α-HPV 16 E7 (Zymed Laboratories). H-RasV12–expressing cells (used as a positive control for soft agar assays) were made by transfecting MCF-10A cells with 10 μg pBabe-H-RasV12 cDNA using Lipofectamine 2000™ (Invitrogen).

Immunoblot analysis

Cells were lysed in Triton X-100 lysis buffer (1% Triton X-100, 50 mM Tris HCl, pH 7.6, 150 mM NaCl, 10 mM NaF, 10 mM β-glycerophosphate, 1mM Na_3VO_3) supplemented with protease inhibitors. Lysates were cleared by centrifugation for 15 min at 4°C. Equivalent amounts of protein were boiled in SDS sample buffer, resolved using SDS-PAGE, and transferred to PVDF membrane. The membranes were blocked in TBST (10 mM Tris HCl, pH 8.0, 150 mM NaCl, 0.1% Tween 20) with 5% nonfat dry milk, and incubated with the primary antibodies indicated overnight at 4°C. Membranes were washed, incubated with HRP-conjugated secondary antibodies, and analyzed by ECL.

Immunostain for GLUT 1 expression

ER-Akt cells were plated on acid-washed 12-mm circular glass coverslips (200,000 cells) and cultured overnight before the addition of OHT. The cells were treated with vehicle control or 1 μM OHT for 24 h, fixed in 100% acetone at room temperature for 15 min, washed three times with PBS/100 mM glycine for 10 min each, and blocked with PBS containing 10% goat serum and 1% BSA. Primary antibodies, α-GLUT-1 (MYM, Chemicon) and α-HA (HA-11, Covance), were diluted at 1:100 in PBS containing 10% GS and incubated overnight at 4°C. Unbound primary antibodies were removed by washing in PBS. Anti–rabbit secondary antibodies coupled with Alexa® Fluor dyes (Molecular Probes) were diluted at 1:200 in PBS containing 10% GS and incubated for 45–60 min. The slips were washed, incubated for 15 min with 0.5 ng/ml DAPI (Roche), mounted with the anti-fade agent Prolong (Molecular Probes), and analyzed using confocal microscopy.

Coculture assays

MEK2DD, ER-Akt, and E7 + ER-Akt–expressing cells were superinfected with pMX-GFP retrovirus to GFP-labeled "producer" cells for tracking purposes. Either MCF10A or HPV E7 "target cells" were labeled with 20 μM Celltracker Orange™ for 30 min at 37°C in serum-free DMEM/F12 before harvest. Each GFP-labeled "producer" cell line was then combined with the orange-labeled "target" cells at producer-to-target ratios of 1:1, 2:1, 5:1, and 9:1 with total cell number kept constant at 6,000 cells/well. The cell mixtures were then 3D cultured on basement membrane with assay media lacking EGF for 20–25 d; 1 μM OHT was added to cultures containing ER-Akt and E7 + ER-Akt "producer" cells at day 3 of culture in order to activate Akt. Indirect immunofluorescence was performed with a Nikon Eclipse TE300 microscope.

Conditioned media experiments

Confluent monolayers of uninfected MCF-10A, MEK2 DD, ER-Akt, and E7 + ER-Akt–expressing cells were washed several times with PBS and then refed with assay media lacking EGF; if appropriate, 1 μM OHT was added to the media to activate Akt. Conditioned media from each cell type was collected 48 h later. The target MCF-10A cells were plated at 20,000 cells/

well in a 12-well dish (Costar) in assay media lacking EGF and allowed to recover for 24 h; subsequently, the media was replaced with freshly harvested conditioned media from the cell types described above. Cells were cultured for 4–6 d, refeeding with freshly harvested conditioned media every 2 d, and examined using crystal violet staining (4% paraformaldehyde, 0.2% crystal violet, PBS).

Soft agar assays

10^4 cells/ml were mixed 1:1 (vol/vol) with 0.6% agarose in 2× MCF-10A growth medium for a final concentration of 0.3% agarose and a final cell number of 5×10^3 per 35-mm well. The cell mixture was plated on top of a solidified layer of 0.6% agarose in 1× growth medium. When indicated, OHT was added at a final concentration of 1 μM. Cells were fed every 4 d with growth medium containing 0.3% agarose, and colonies were photographed at 30 d post-seeding.