Supporting Information

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SI Materials and Methods

Materials. DAPI, brefelden A, mitomycin C, and Exo1 were obtained from Calbiochem. T101 was purchased from Zedira. The rhodamine-conjugated phalloidin, EGF, Lipofectamine, Lipofectamine 2000, protein G beads, control and tTG siRNAs, and all cell-culture reagents were from Invitrogen. The fibronectin (FN) antibody, monodansylcadaverine (MDC), and biotinylated pentylamine (BPA) were from Sigma. The tissue transglutaminase (tTG) and actin antibodies were obtained from Lab Vision/Thermo. Flotillin-2 antibody was obtained from Santa Cruz, and HA and Myc antibodies were from Covance. The Steriflip PVDF filters (0.45-μm pore size) were from Millipore. The antibodies against IκBα and GFP and antibodies that recognize ERK, protein kinase B (PKB/AKT), focal adhesion kinase (FAK), and the EGF receptor were from Cell Signaling.

Cell Culture. The MDAMB231, U87, MCF10A, and HeLa cell lines were grown in RPMI-1640 medium containing 10% FBS. The NIH 3T3 cell line was grown in DMEM containing 10% calf serum (CS). Expression constructs were transfected into cells using Lipofectamine. Control and tTG siRNAs were introduced into cells with Lipofectamine 2000. As indicated, cells were incubated with serum-free medium containing combinations of 0.1 μg/mL EGF, 100 μM MDC, 10 μM T101, 10 μM BFA, and 10 μM Exo1. To arrest MDAMB231 cells mitotically, plates of cells were treated with 10 μg/mL mitomycin C for 2 h; then the solution was rinsed away and the cells were allowed to recover in growth medium (RPMI-1640 medium containing 10% FBS) for 1 d.

Isolation of Microvesicles from Cancer Cells. For each of the experiments that used microvesicle (MV) preparations, the conditioned medium from 5.0×10^6 serum-starved MDAMB231 cells or U87 cells (which is the equivalent of two nearly confluent 150-mm dishes of either of these cell lines) were collected, and the MV isolated were from the medium as previously described (1, 2). Briefly, the conditioned medium was subjected to two consecutive centrifugations; the first at $300 \times g$ for 10 min pelleted intact cells, and the second at $12{,}000 \times g$ for 20 min pelleted cell debris. To generate MV lysates, the conditioned medium was centrifuged a third time at $100{,}000 \times g$ for 2 h, and the resulting pellet was washed with PBS and then lysed in 250 μL cell lysis buffer (25 mM Tris, 100 mM NaCl, 1% Triton X-100, 1 mM EDTA, 1 mM DTT, 1 mM $NaVO_4$, 1 mM β-glycerol phosphate, and 1 μg/mL aprotinin). To generate intact MV for the cell-based assays and other experiments as indicated, the partially purified conditioned medium (medium cleared of cells and cell debris) was filtered using a Millipore Steriflip PVDF filter with a 0.45-μm pore size. The MV retained by the PVDF membrane were resuspended in serum-free medium, with each preparation yielding enough MV to treat two or three wells of a six-well dish of recipient cells for a given experiment.

Immunoblot Analysis and Immunoprecipitation. The protein concentrations of the whole-cell lysates (WCL) were determined using the Bio-Rad DC protein assay. The MV lysates were normalized for comparison by isolating them from the conditioned medium of 5.0×10^6 serum-starved MDAMB231 cells or U87 cells for each of the experimental conditions assayed and then by lysing in 250 μL of cell lysis buffer. For immunoprecipitations, equal volumes of MV lysates or 300 μg of WCL were incubated with a tTG antibody and protein G beads. The bead–antibody–protein complexes collected by centrifugation, as well as the WCL (40 μg) and MV extracts (75 μL), were resolved by SDS/PAGE, and the proteins were transferred to PVDF membranes. The filters were incubated with the indicated primary antibodies diluted in 20 mM Tris, 135 mM NaCl, and 0.02% Tween 20 (TBST). The primary antibodies were detected with HRP-conjugated secondary antibodies (Amersham Biosciences) followed by exposure to ECL reagent.

Immunofluorescence. Cells were fixed with 3.7% paraformaldehyde and then some samples were permeabilized with PBS containing 0.1% Triton X-100. Permeabilized and nonpermeabilized samples were incubated with a tTG antibody and then were incubated with Oregon green 488-conjugated secondary antibody. Rhodamine-conjugated phalloidin was used to stain actin, and DAPI was used to stain nuclei. The cells were visualized by fluorescent microscopy, and the images were captured and processed using IPLABS.

Live Image Fluorescence Microscopy. MDAMB231 cells transiently expressing GFP-PM, a GFP-tagged form of the plasma membrane targeting sequence in Lyn, were visualized by fluorescent microscopy. Images of the transfectants were captured in 30-s intervals over a span of 15 min.

Transamidation Assay. The transamidation activity in whole-cell extracts was read out by the incorporation of BPA into lysate proteins as previously described (3). The transamidation activity of recombinant tTG (0.1 μM) exposed to increasing concentrations of T101 was determined using a spectrophotometric assay (4). The transamidation activity associated with MV was read out by incubating equal amounts (75 μL) of each MV sample in a buffer containing 40 mM N′*N*-dimethyl casein, 2 mM BPA, 40 mM $CaCl_2$, and 40 mM DTT for 15 min. The reaction was stopped by the addition of Laemmli sample buffer followed by boiling. The reactions then were resolved by SDS/PAGE, and the proteins were transferred to PVDF membranes. The filters were blocked with 100 mM boric acid, 20 mM sodium borate, 0.01% SDS, 0.01% Tween 20, and 80 mM NaCl (BBST) containing 10% BSA and then were incubated with HRP-conjugated streptavidin diluted in BBST containing 5% BSA for 1 h at room temperature, followed by extensive washing with BBST. The incorporation of BPA into N′*N*-dimethyl casein was visualized after the membranes were exposed to ECL reagent.

Scanning Electron Microscopy and Immuno-Scanning Electron Microscopy. MDAMB231 cells grown on Lab-Tek chamber slides (Nunc) were fixed for 1 h with 2% electron microscopy (EM)-grade glutaraldehyde diluted in a 0.05 M cacodylic acid buffer solution (pH 7.4). For immuno-scanning electron microscopy (immuno-SEM), filter-isolated MDAMB231 cell-derived MV were added to Lab-Tek chamber slides, allowed to attach, and then were fixed for 1 h with 2% EM-grade glutaraldehyde in PBS. After blocking for 15 min in 0.1 M glycine and for an additional 30 min in PBS containing 5% BSA, 0.1% gelatin, and 5% goat serum, the MV were incubated for 1 h with the tTG antibody diluted in PBS (2 μg/mL). After washing with PBS, the MV samples were incubated for 1 h with 6-nm gold particle-conjugated goat anti-mouse IgG (Electron Microscopy Sciences) diluted in PBS. Both the cell and the immuno-labeled MV samples were postfixed for 1 h with 1% osmium tetroxide in PBS and were dehydrated in graded ethanol solutions of 25%, 50%, 70%, 95%, and 100% ethanol before being placed in a CPD-30 critical point drying machine (SCD050; BAL-TEC). The cells then were sputter-coated with platinum, and the immuno-labeled MV were sputter-coated with amorphous carbon before

being observed with a Leo 1550 field-emission scanning electron microscope.

Cell Growth Assays. NIH 3T3 cells were plated in each well of a six-well dish at a density of 10×10^4 cells per well and were maintained in DMEM containing 2% CS supplemented with or without MV derived from 5.0×10^6 MDAMB231 cells or U87 cells. Once a day for 3 d, one set of cultures was collected and counted; in the remaining sets of cells the culturing medium was replenished (including the addition of freshly isolated MV). The assays were performed three times, and the results were averaged together and graphed.

Anchorage-Independent Growth Assays. Parental NIH 3T3 cells or MCF10A cells incubated without or with MV derived from 5.0×10^6 MDAMB231 cells or U87 cells, NIH 3T3 cells stably overexpressing the vector control, wild-type tTG, or Cdc42 F28L cells were plated at a density of 7×10^3 cells/mL in medium containing 0.3% agarose, with or without various inhibitors as indicated, onto underlays composed of growth medium containing 0.6% agarose in six-well dishes. The soft agar cultures were re-fed (including the addition of freshly prepared MV and treatment with various inhibitors as indicated) every third day for 12 d, at which time the colonies that had formed were counted. Each of the assays was performed at least three times, and the results were averaged together and graphed.

Cell Death Assays. NIH 3T3 cells or MCF10A cells were plated in each well of a six-well dish and then were cultured in medium containing 2% CS or serum-free medium supplemented with or without MV derived from 5.0×10^6 MDAMB231 cells or U87 cells and with or without MDC or T101, as indicated. Two days later the cultures were fixed and stained with DAPI for viewing by fluorescence microscopy. Cells undergoing apoptosis were identified by nuclear condensation or blebbing, and the percentage of cell death was determined by calculating the ratio of apoptotic cells to total cells for each condition. These experiments were conducted at least three times, and the results from each experiment were averaged together and graphed.

Flow Cytometry. Intact MV were isolated from the conditioned medium of 5.0×10^6 mock-transfected MDAMB231 cells or MDAMB231 cells transiently expressing GFP using the filter method (described above) and were resuspended in PBS containing 0.1% BSA. The MV samples were evaluated using a BD LSR II flow cytometer by gating events that were~1–3 μm in size and determining whether they expressed GFP. At least 500 events were collected for each sample, and the data were analyzed using BD FACSDiva software. The experiments were performed at least three times; similar results were obtained from each experiment.

Proteomic Analyses. Lysates of MDAMB231 cell- and U87 cell-derived MV (~30 μg of each sample) were resolved by SDS/PAGE and then stained using the Colloidal Blue Staining Kit (Invitrogen) according to the manufacturer's protocol. The proteins were excised from the gel and digested with trypsin. The resulting protein samples were analyzed at the Cornell Proteomic Facility using a triple quadrupole linear ion trap (4000 Q Trap) on-line LC/MS/MS system (Applied Biosystems/MDS Sciex) or the Synapt HDMS system (Waters). Protein identification was achieved by performing peptide alignment searches against the NCBI RefSeq protein database.

Mouse Studies. We combined 5×10^5 mitotically arrested (using mitomycin C) MDAMB231 cells stably expressing control or tTG siRNAs with 5×10^5 NIH 3T3 fibroblasts and growth factor-reduced Matrigel (BD Biosciences) to achieve 30% Matrigel in the final solution. The cell preparations were injected s.c. into the flanks of 6- to 8-wk-old female NIH-III nude mice. As controls, parental MDAMB231 cells and NIH 3T3 cells (5×10^5 cells of each cell line) were singly combined with growth factor-reduced Matrigel (to a final concentration of 30% Matrigel) and then were injected into mice as well. After 1 mo the animals were killed, and the resulting tumors that had formed for each experimental condition were excised and counted. The experiments involving mice were performed in accordance with the protocols approved by the Cornell Center for Animal Resources and Education.

1. Skog J, et al. (2008) Glioblastoma microvesicles transport RNA and proteins that promote tumour growth and provide diagnostic biomarkers. *Nat Cell Biol* 10:1470–1476.
2. Al-Nedawi K, et al. (2008) Intercellular transfer of the oncogenic receptor EGFRvIII by microvesicles derived from tumour cells. *Nat Cell Biol* 10:619–624.
3. Antonyak MA, et al. (2004) Augmentation of tissue transglutaminase expression and activation by epidermal growth factor inhibit doxorubicin-induced apoptosis in human breast cancer cells. *J Biol Chem* 279:41461–41467.
4. Datta S, Antonyak MA, Cerione RA (2006) Importance of Ca(2+)-dependent transamidation activity in the protection afforded by tissue transglutaminase against doxorubicin-induced apoptosis. *Biochemistry* 45:13163–13174.

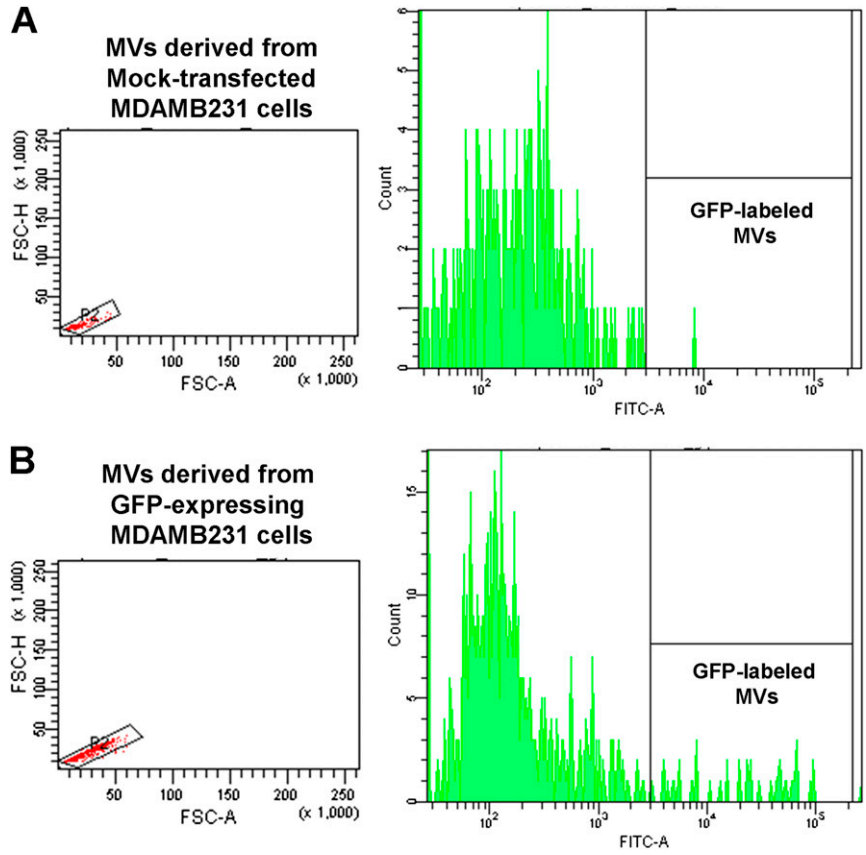


Fig. S1. MV are constitutively released by MDAMB231 breast cancer cells into their culturing medium. MDAMB231 cells that were mock transfected or transfected with a plasmid encoding GFP (pEGFP) were placed in serum-free medium for 1 d. The conditioned medium from the transfectants was collected, and the intact MV present in the medium were isolated and subjected to FACS analysis by gating for GFP-positive MV ~1–3 μm in diameter. (*A*) The results obtained when the MV isolated from the mock-transfected MDAMB231 cells were analyzed. (*B*) The results obtained when the MV isolated from MDAMB231 cells transiently expressing GFP were analyzed.

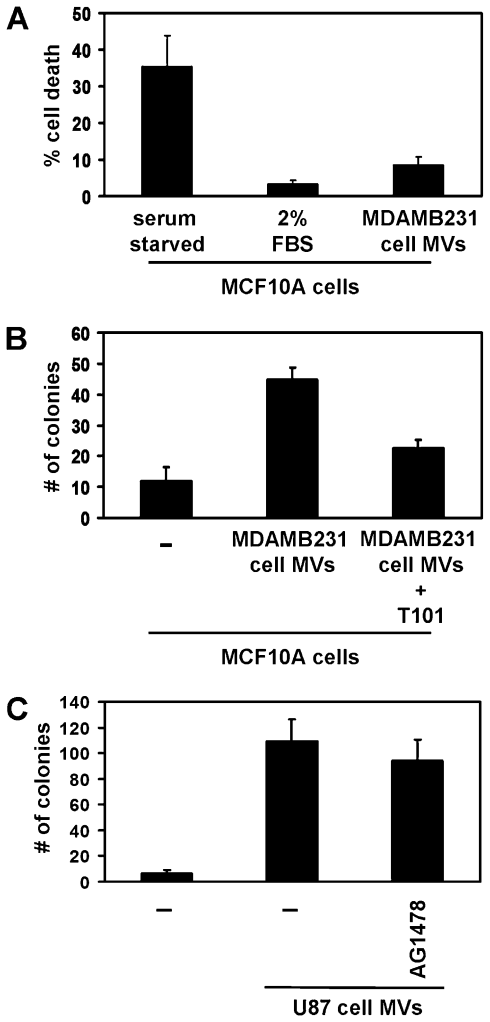


Fig. S2. MDAMB231 cell-derived MV transform recipient MCF10A mammary epithelial cells. (*A*) Cell death assays were performed on MCF10A cells that were cultured for 3 d in serum-free medium, medium containing 2% FBS, or serum-free medium supplemented with intact MV derived from 5.0×10^6 MDAMB231 cells. (*B*) Anchorage-independent growth assays were performed on MCF10A cells incubated with MV derived from 5.0×10^6 MDAMB231 cells treated with or without the tTG inhibitor T101. (*C*) Anchorage-independent growth assays were performed on NIH 3T3 fibroblasts incubated with U87 cell-derived MV treated with or without the EGF receptor inhibitor AG1478. The culturing medium (including the MV, T101, and AG1478) for the soft agar assays performed in *B* and *C* was replenished every third day for 12 d, at which time the colonies that had formed were counted. The data shown in *A*, *B*, and *C* represent the mean ± SD from three independent experiments.

Proteomic analyses of microvesicles shed by MDAMB231 cells and U87 cells

Nucleic Acid-binding Proteins
eukaryotic translation elongation factor 1
eukaryotic translation elongation factor 2
histone cluster 1
histone cluster 2
RuvB-like protein 1
RuvB-like protein 2

Extracellular Matrix and Plasma Membrane-associated Proteins
annexin A2
CD9 antigen
collagen
Ecto-5'-nucleotidase
EGF-like repeats and discoidin I-like domains-containing protein 3
fibronectin
galectin 3 binding protein
integrin beta 1
laminin
lysyl hydroxylase precursor
major histocompatibility complex
Na+/K+ -ATPase
transglutaminase 2 isoform a

Metabolic Proteins
aldolase A
enolase
ferritin
glyceraldehyde-3-phosphate dehydrogenase
L-lactate dehydrogenase A
nicotinamide phosphoribosyltransferase precursor
phosphoglycerate kinase 1
pyruvate kinase
UDP-glucose pyrophosphorylase

Cytoskeletal Proteins
actin
actinin
chaperonin
moesin
T-complex protein 1
tubulin
vimentin

Signaling,Trafficking, and other functional proteins
adenylyl cyclase-associated protein
alpha-2-macroglobulin precursor
heat shock protein 70kDa
heat shock protein 90kDa
HtrA serine peptidase 1 precursor
valosin-containing protein

Fig. S3. Proteins common to both MDAMB231 cell- and U87 cell-derived MV. Proteomic analysis was performed on MV shed by MDAMB231 breast cancer cells or by U87 brain tumor cells. A list was compiled (based on general cellular function) of proteins identified in the MV from both MDAMB231 and U87 cells.

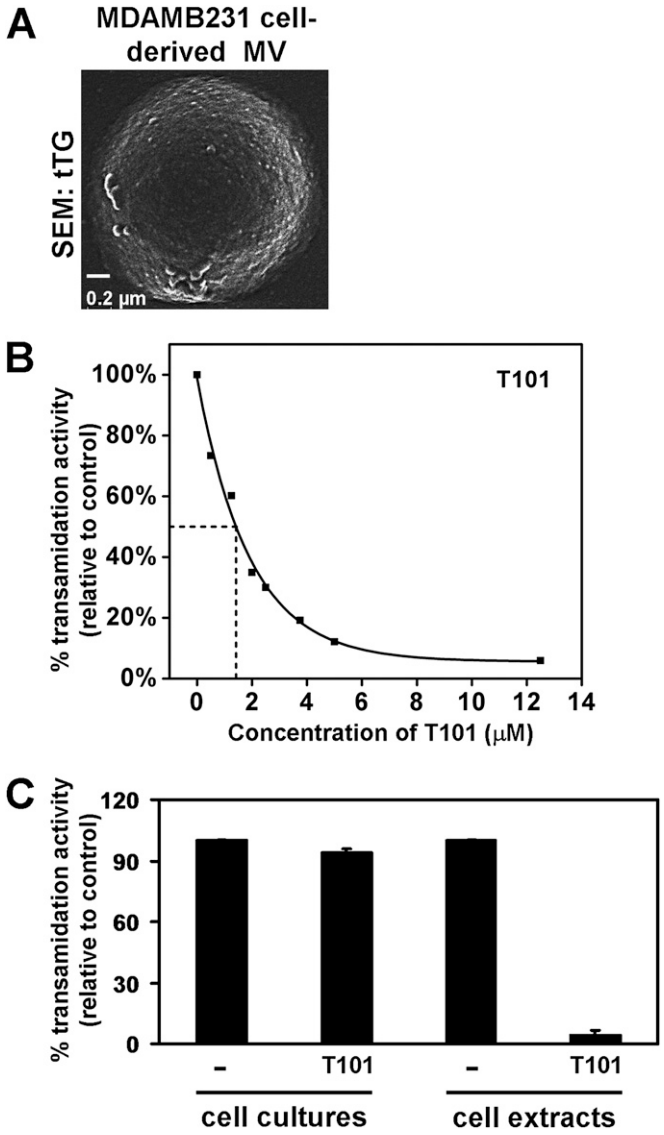


Fig. S4. tTG is localized to the outer leaflet of MV membranes. (*A*) Intact MDAMB231 cell-derived MV were isolated, fixed, immunostained with a tTG antibody, and then processed for detection by SEM. Shown is a representative SEM image of an MV. Note the detection of tTG on the surface of the MV. (*B*) The transamidation activity of a fixed concentration (1 μM) of purified recombinant tTG incubated with increasing concentrations of the tTG inhibitor T101 was assayed. The IC_{50} of T101 (dashed lines) was determined to be ~1.5 μM. This experiment was repeated two additional times, with comparable results. (*C*) Transamidation activity assays, as read out by the incorporation of BPA into lysate proteins, were performed on the cell extracts of MDAMB231 cells that had been cultured in medium supplemented with or without 200 μM T101 (a 133-fold greater concentration than the IC_{50} calculated for this inhibitor in *B*) for ~10 h before being washed extensively and then lysed (cell cultures). Equal amounts of an MDAMB231 cell extract were left untreated or were incubated with 10 μM T101 15 min before being subjected to a transamidation activity assay (cell extracts). Data are mean ± SD from three independent experiments.

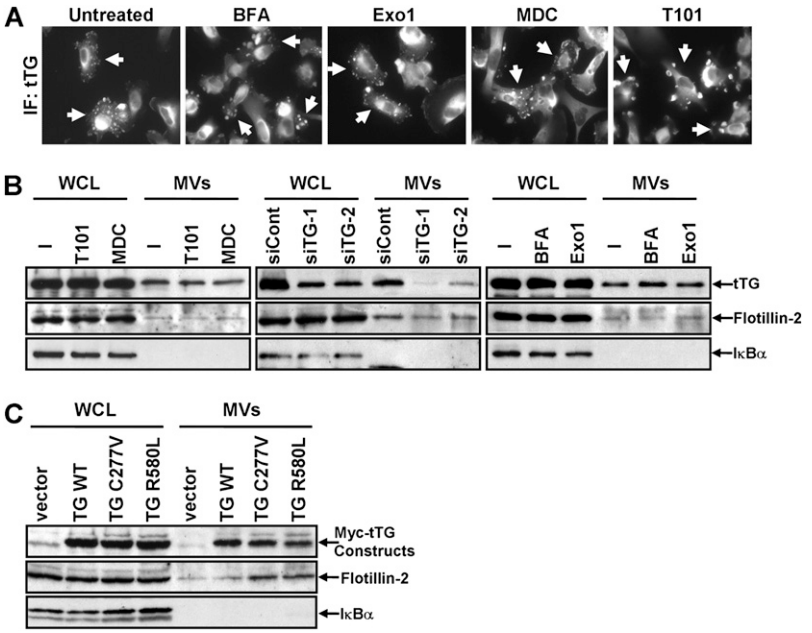


Fig. S5. tTG is not important for the ability of human cancer cells to generate MV. (*A*) Serum-starved MDAMB231 cells treated with or without T101, MDC, BFA, or Exo1 were immunostained with a tTG antibody. Shown are representative images of the cells exposed to the various inhibitors. Cells forming MV are indicated by arrows. (*B*) Serum-starved MDAMB231 cells treated with or without the tTG inhibitors T101 and MDC (*Left*), transfected with either control siRNA (siCont) or two distinct tTG siRNAs (siTG-1 and siTG-2) (*Center*), or treated with or without the inhibitors of classical secretion BFA and Exo1 (*Right*) were lysed (WCL), and the MV released into the medium by the cells also were collected and lysed. The extracts were immunoblotted with antibodies against tTG, the MV marker flotillin-2, and the cytosolic-specific marker IκBα. (*C*) WCL of MDAMB231 cells ectopically expressing vector only or Myc-tagged forms of wild-type tTG (TG WT), a transamidation-defective form of tTG (TG C277V), or a GTP-binding–defective form of tTG (TG R580L), as well as lysates of the MV shed by the transfectants, were immunoblotted with antibodies against the Myc tag, flotillin-2, and IκBα.

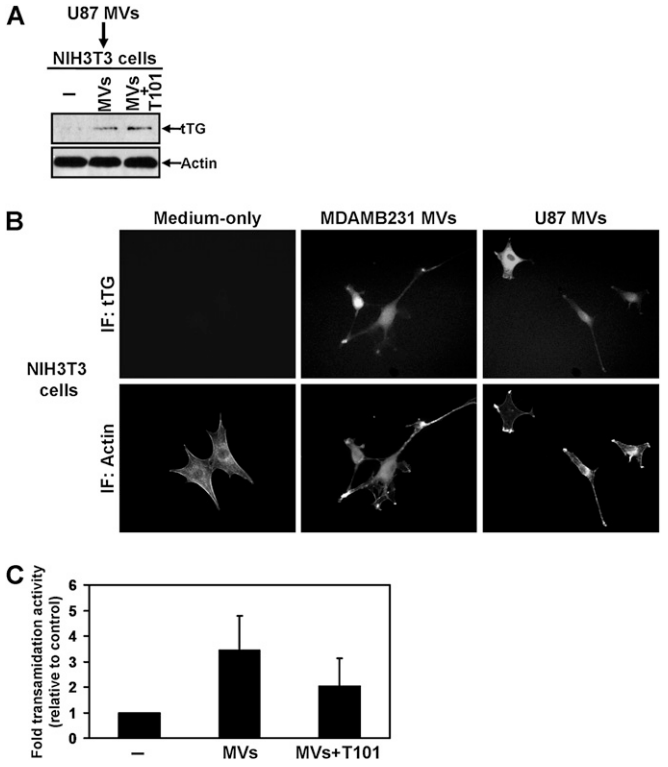


Fig. S6. tTG is transferred from U87 cells to recipient fibroblasts in MV. (*A*) Lysates of fibroblasts incubated for 30 min with U87 cell-derived MV that had been pretreated with or without the cell-impermeable tTG inhibitor T101 were immunoblotted with tTG and actin antibodies. (*B*) NIH 3T3 cells incubated for 30 min with serum-free medium supplemented with or without intact MV generated by either MDAMB231 or U87 cells were immunostained with a tTG antibody and rhodamine-conjugated phalloidin to detect actin. Shown are representative fluorescent images of the fibroblasts. Note that tTG is detected only in the fibroblasts incubated with cancer cell-derived MV. (*C*) Lysates of fibroblasts incubated for 30 min with U87 cell-derived MV that had been pretreated with or without T101 were assayed for transamidation activity as read out by the incorporation of BPA into lysate proteins.

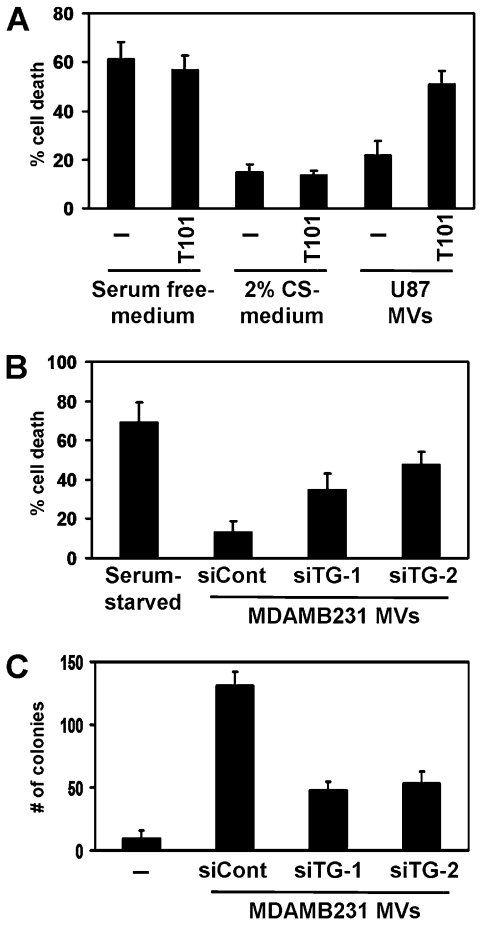


Fig. S7. tTG is important for mediating the survival and aberrant growth advantages afforded to cells incubated with cancer cell-derived MV. (*A*) Cell death assays were performed on fibroblasts maintained in serum-free medium, 2% CS medium, or serum-free medium containing MV derived from 5.0×10^6 U87 glioma cells. Each culturing medium was supplemented further with the cell-impermeable tTG inhibitor T101 or was untreated. (*B* and *C*) The MV shed from 5.0×10^6 serum-starved MDAMB231 cells transfected with control siRNA (siCont) or two different tTG siRNAs (siTG-1 and siTG-2) were collected and re-suspended in serum-free DMEM. (*B*) NIH 3T3 cells plated in each well of a six-well dish then were placed in serum-free medium or in serum-free medium containing the different MV preparations for ~35 h, at which time the cell death rates of the different cell cultures were determined. (*C*) Anchorage-independent growth assays were performed on NIH 3T3 fibroblasts incubated with the different MV preparations outlined above. The soft agar cultures were re-fed (including the addition of freshly prepared MV) every third day for 12 d, at which time the colonies that had formed were counted. The data shown in *A*, *B*, and *C* represent the mean ± SD from at least three independent experiments.

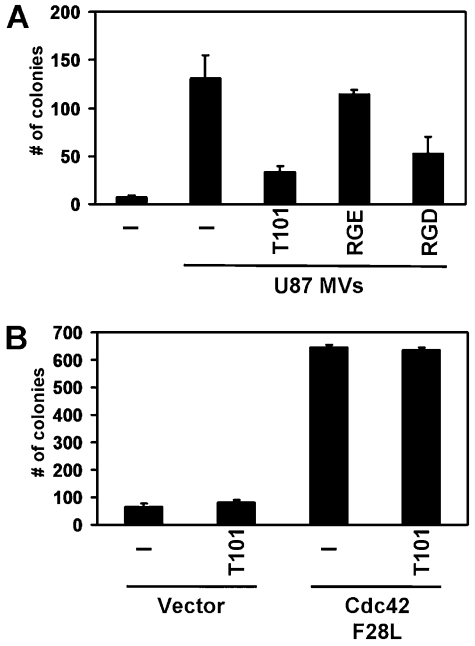


Fig. S8. The transforming properties of U87 cell-derived MV require tTG activity. (*A*) Anchorage-independent growth assays were performed on control NIH 3T3 fibroblasts or on fibroblasts incubated with MV derived from 5.0×10^6 U87 glioma cells treated with T101, treated with the arginine-glycine-aspartic acid (RGD) peptide, the arginine-glycine-glutamic acid (RGE) control peptide, or untreated. (*B*) Anchorage-independent growth assays also were performed on NIH 3T3 cells stably expressing vector alone or an activated form of Cdc42 (Cdc42 F28L) treated with 200 μM T101 or untreated cells. Note that the ability of Cdc42 F28L to induce colony formation is insensitive to T101. The data shown in *A* and *B* represent the mean ± SD from at least three independent experiments.

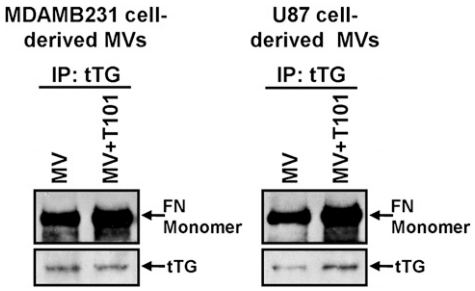
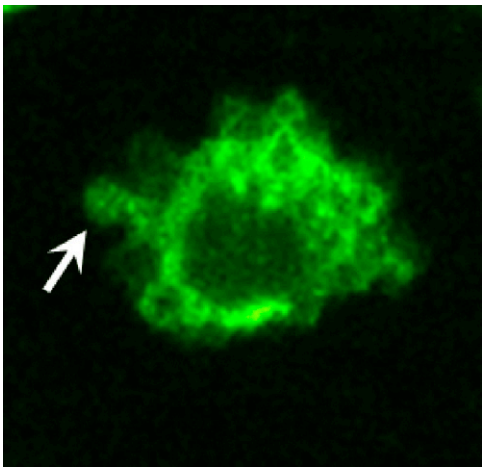


Fig. S9. T101 does not interfere with the ability of tTG to associate with FN in MDAMB231 cell- or U87 cell-derived MV. Intact MV collected from MDAMB231 or U87 cells were treated with T101 or were left untreated before being lysed. MV extracts then were subjected to immunoprecipitation using a tTG antibody (IP: tTG). The resulting immunocomplexes were immunoblotted with FN and tTG antibodies.



Movie S1. MDAMB231 cells transiently expressing GFP-PM (to label the plasma membranes of the transfectants) were subjected to live-imaging fluorescent microscopy. Highlighted with an arrow is a GFP-labeled MV being shed by a cell.

Movie S1