Supplemental Data

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The Epithelial-Mesenchymal Transition Generates Cells with Properties of Stem Cells

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Supplemental Experimental Procedures

Cell Culture

The HMLE-Snail-ER and HMLE-Twist-ER cells were generated by infecting the HMLE cells with pWZL-Snail-ER or pWZL-Twist-ER vectors followed by selection with 5 ng/ml of blasticidin. HMLE-Snail-Ras, HMLE-Twist-Ras, and HMLE-Vector-Ras cells were generated by infecting immortalized human mammary epithelial cells (Elenbaas et al., 2001) with retroviral vectors expressing Snail or Twist or the control vector and selecting with 2μg/ml puromycin. These cells were then transformed by introduction of a pWZL retroviral vector expressing the V12H-RAS oncogene followed by selection with 4μg/ml of blasticidin.

Mammosphere culture

Mammosphere culture was performed as described in Dontu et al 2003 (Dontu et al., 2003), except that the culture medium contained 1% methyl cellulose to prevent cell aggregation. The mammospheres were cultured for 7-10 days. Then the mammospheres with diameter >75 μm were counted. For serial passages, mammosphere were harvested using 70 μm cell strainers after culturing mammospheres for 10 days. The mammospheres were dissociated to single cells with trypsin, and 500 dissociated cells were plated in a 96-well plate and cultured for 10 days. The mammospheres were then counted. The individual mammospheres were found to be derived from single cells, because mixing of equal numbers of DiI (Red)- or DiO (Green)-labelled cells prior to performing the mammospheres containing only one or the other label.

Plasmids, virus production and infection of target cells

The development of pBp-Snail and pBp-Twist was reported earlier (Yang et al., 2004). The pWZL-Blast-Snail-ER and PWZL-Blast-Twist-ER constructs were generated by replacing the *MYC* cDNA of pWZL-Blast-DN-MycER (Littlewood et al., 1995; Watnick et al., 2003) with the hSnail cDNA PCR amplified from pBp-hSnail or the mTwist cDNA PCR-amplified from pBp-mTwist. The production of lentiviruses and amphotropic retroviruses as well as the infection of target cells was described previously (Stewart et al., 2003).

Primers used to amplify the genes:

hFOXC2-5 GCCTAAGGACCTGGTGAAGC

hFOXC2-3 TTGACGAAGCACTCGTTGAG

hGAPDH-5 ACCCAGAAGACTGTGGATGG

hGAPDH-3 TCTAGACGGCAGGTCAGGTC

hEcad-5 TGCCCAGAAAATGAAAAAGG

hEcad-3 GTGTATGTGGCAATGCGTTC

hNcad-5 ACAGTGGCCACCTACAAAGG

hNcad-3 CCGAGATGGGGTTGATAATG

hFN1-5 CAGTGGGAGACCTCGAGAAG

hFN1-3 TCCCTCGGAACATCAGAAAC

hVim-5 GAGAACTTTGCCGTTGAAGC

hVim-3 GCTTCCTGTAGGTGGCAATC

hSnail-5 CCTCCCTGTCAGATGAGGAC

hSnail-3 CCAGGCTGAGGTATTCCTTG

hTwist-5 GGAGTCCGCAGTCTTACGAG

hTwist-3 TCTGGAGGACCTGGTAGAGG

hSIP1-5 TTCCTGGGCTACGACCATAC

hSIP1-3 TGTGCTCCATCAAGCAATTC

hSlug-5 GGGGAGAAGCCTTTTTCTTG

hSlug-3 TCCTCATGTTTGTGCAGGAG

Immunofluorescence

About $2.5x10^4$ cells were seeded on a 4-well Lab-TekII Chamber Slide. After 24 hrs, the cells were washed with phosphate-buffered saline (PBS) twice and permeabilized and fixed in 2% paraformaldehyde and 0.1% Triton X100 in PBS buffer at 4^0C for 30 minutes. The cells were then washed 3 times with PBS and incubated with the blocking solution (10% goat serum in PBS). The cells were then incubated with the primary antibodies for (2 hr to overnight), washed 3 times with PBS plus 0.1% Tween-20 for 15 minutes, and finally incubated with secondary antibodies (Invitrogen) and DAPI for 2 hours. The slides were washed extensively with PBS and mounted with slow fade Light Anti fade Kit (Invitrogen). All matched samples were photographed (control and test) using immunofluorescence microscope and identical exposure times.

Mammospheres and the mammospheres differentiated in Matrigel were cryo-embedded in OCT compound (Sakura Tissue-TEK) and sectioned at 8-μm thickness. The sections were stained with antibodies against cytokeratin 14 (AF64, Covance), Cytokeratin 18 (DC10, NeoMarkers), Cytokeratin 8 (Troma-1, Developmental Studies Hybridoma Bank), CD49f/integrin α6 (GoH3, Pharmingen), and Muc1 (HMPV, Pharmingen).

Supplemental References

Dontu, G., Abdallah, W.M., Foley, J.M., Jackson, K.W., Clarke, M.F., Kawamura, M.J., and Wicha, M.S. (2003). In vitro propagation and transcriptional profiling of human mammary stem/progenitor cells. Genes Dev *17*, 1253-1270.

Littlewood, T.D., Hancock, D.C., Danielian, P.S., Parker, M.G., and Evan, G.I. (1995). A modified oestrogen receptor ligand-binding domain as an improved switch for the regulation of heterologous proteins. Nucleic Acids Res *23*, 1686-1690.

Stewart, S.A., Dykxhoorn, D.M., Palliser, D., Mizuno, H., Yu, E.Y., An, D.S., Sabatini, D.M., Chen, I.S., Hahn, W.C., Sharp, P.A., *et al.* (2003). Lentivirus-delivered stable gene silencing by RNAi in primary cells. Rna *9*, 493-501.

Watnick, R.S., Cheng, Y.N., Rangarajan, A., Ince, T.A., and Weinberg, R.A. (2003). Ras modulates Myc activity to repress thrombospondin-1 expression and increase tumor angiogenesis. Cancer Cell *3*, 219-231.

Yang, J., Mani, S.A., Donaher, J.L., Ramaswamy, S., Itzykson, R.A., Come, C., Savagner, P., Gitelman, I., Richardson, A., and Weinberg, R.A. (2004). Twist, a master regulator of morphogenesis, plays an essential role in tumor metastasis. Cell *117*, 927-939.

Table S1

Starting population	End Population (%)	Days in culture				
		0	2	4	6	8
$CD44^{high}/CD24^{low}$	**$CD44^{low}/CD24^{high}$**	0	**20**	**30**	**60**	**71**
	$CD44^{high}/CD24^{low}$	100	80	70	47	29
$CD44^{low}/CD24^{high}$	**$CD44^{low}/CD24^{high}$**	100	100	99	100	100
	$CD44^{high}/CD24^{low}$	0	0	1	0	0

In vitro passaging in monolayer culture of $CD44^{high}/CD24^{low}$ HMLE cells resulted in the appearance of $CD44^{low}/CD24^{high}$ cells within 2 days. However, *in vitro* passaging in monolayer culture of $CD44^{low}/CD24^{high}$ cells did not result in the appearance of $CD44^{high}/CD24^{low}$ cells, even after 8 days.

Table S2

Starting populations		$CD44^{high}/CD24^{low}$	
End populations		$CD44^{high}/CD24^{low}$	$CD44^{low}/CD24^{high}$
Clone number	1	0.16	98.1
	2	0.02	97.9
	3	0.10	93.8
	4	19.9	56.7
	5	0.00	99.5
	6	0.09	92.0
	7	0.76	68.1
	8	0.26	84.3
	9	0.42	67.7
	10	0.20	86.5
	11	9.19	49.8
	12	41.9	7.31
	13	0.10	95.9

Analysis of single-cell clones derived from a $CD44^{high}/CD24^{low}$ HMLE population. We fractionated HMLE cells into $CD44^{high}/CD24^{low}$ and $CD44^{low}/CD24^{high}$ subpopulations by FACS. We then generated single-cell-derived clones by introducing the $CD44^{high}/CD24^{low}$ cells into 96-well plates at a dilution yielding an average of <1 cell per well. We examined these wells under the microscope to ensure that at most one cell was present in each well.

After culturing for 3 weeks, 12 out of 13 cell populations derived from single-cell clones arising from $CD44^{high}/CD24^{low}$ cells contained a subpopulation of $CD44^{high}/CD24^{low}$ cells and a majority of $CD44^{low}/CD24^{high}$ cells. Table entries indicate the percentage of cells having one or the other immunophenotype; because of gating thresholds used for the FACS analysis, the sum of the two subpopulations within a given clone often do not equal 100%.

Table S3

Number of cells injected	**Reconstituted mammary glands/number of mammary fat pads injected**	
	Mammosphere culture	2D culture
2X10^4	5/6 (83%)	3/6 (50%)
2X10^3	3/6 (50%)	0/6 (0%)
2X10^2	0/6 (0%)	0/6 (0%)

Mammosphere culture enriches for mammary fat-pad-reconstituting stem cells. The relative efficiencies of primary mouse mammary epithelial cells propagated in either 3D (mammosphere) or 2D (monolayer) culture to reconstitute the mammary gland following injection in limiting dilutions into cleared mouse mammary fat pads are shown.

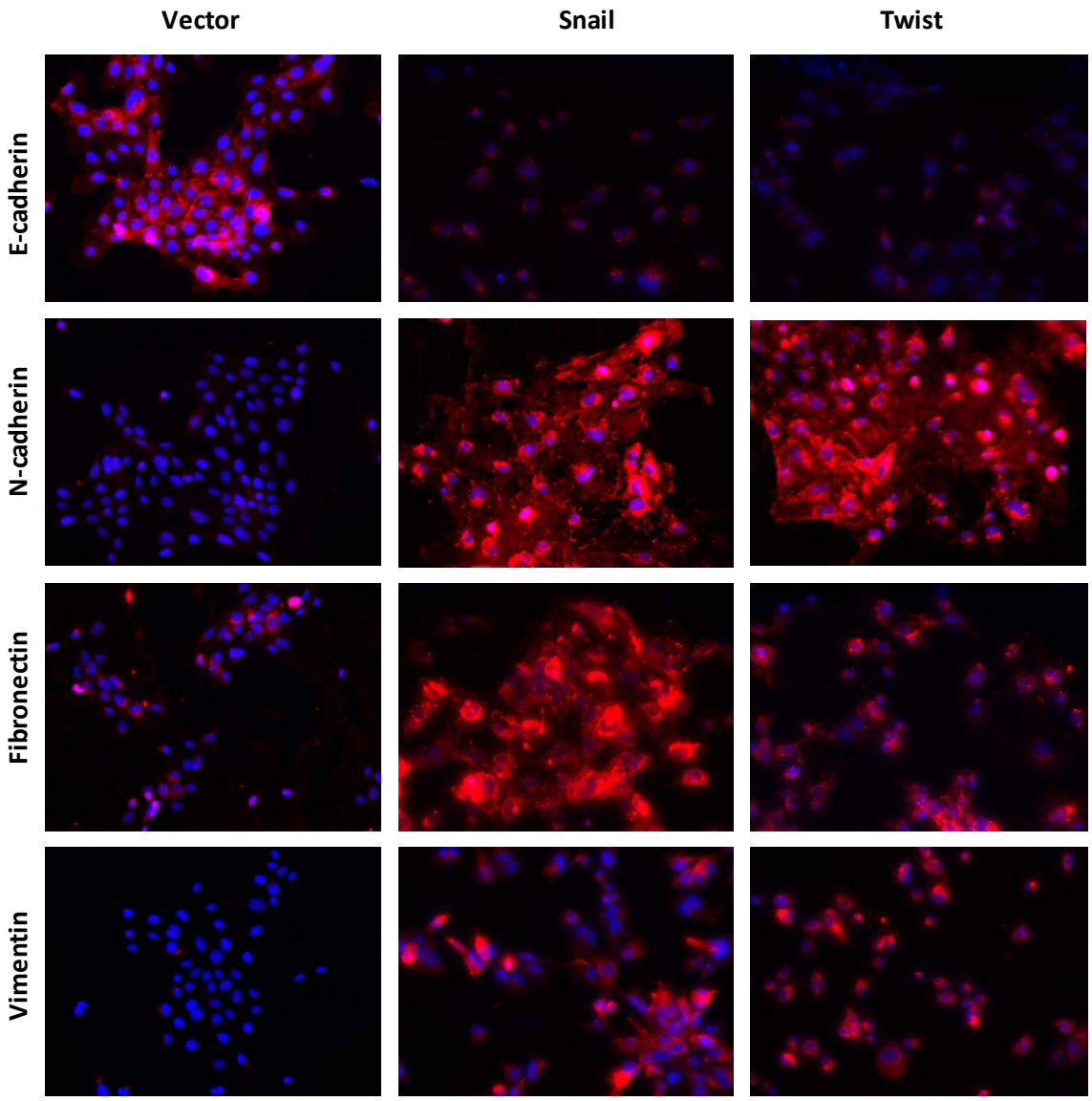
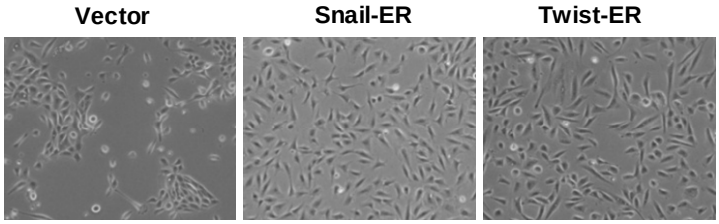
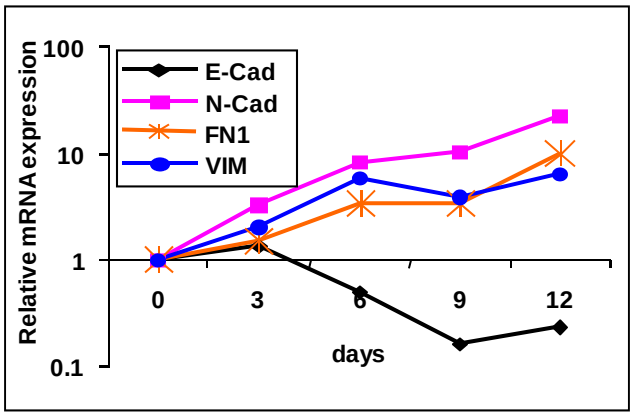


Figure S1. Images of HMLE cells, which underwent an EMT as a result of ectopic expression of Snail or Twist, were immunostained using antibodies against E-Cadherin, N-cadherin, fibronectin or vimentin proteins. Corresponding images of cells expressing control vector are also shown.

A



B



C

	Percentage of CD44^{hi}/CD24^{low} cells following Tamoxifen treatment				
Days	0	3	6	9	12
Vector	1.38	1.44	2.34	1.99	2.36
Snail-ER	0.15	0.62	1.80	14.30	13.20
Twist-ER	0.16	0.10	2.20	21.60	21.50

D

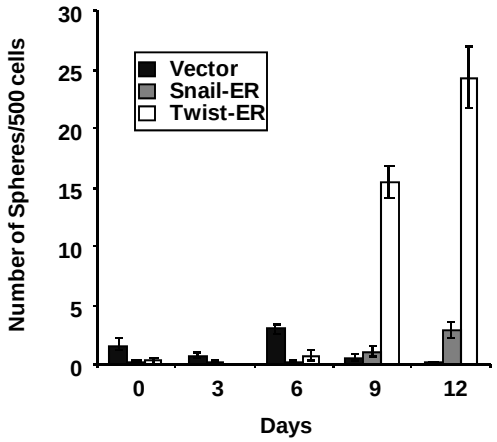


Figure S2. Induction of EMT by either Snail-ER or Twist-ER tamoxifen-inducible vectors generates cells with stem-cell properties (**A**) Phase-contrast images of Snail-ER, Twist-ER or control vector cells treated with tamoxifen for 12 days. (**B**). Expression levels of mRNAs encoding proteins associated with an

EMT, as observed in HMLE cells induced to undergo EMT by ectopic expression of Snail-ER (tamoxifen-induced). The expression levels are reported relative to Day 0 of the 12-day tamoxifen treatment. GAPDH mRNA was used to normalize variability in template loading. The data are reported as mean +/- SEM. (**C**). In an HMLE cell population, $CD44^{high}/CD24^{low}$ cells progressively appeared in parallel with the ongoing induction of EMT by Snail-ER or Twist-ER. (**D**). Snail-ER and Twist-ER cells were treated with tamoxifen to induce EMT and their ability to form mammospheres was measured at multiple time points. Mammosphere assays were performed after ceasing tamoxifen treatment. The number of spheres formed/500 seeded cells is reported as mean +/- SD. The 12-day tamoxifen induction increased mammosphere-forming ability of Snail-ER cells by 10 fold ($P<0.01$) and that of Twist-ER cells by 81 fold ($P<0.001$).

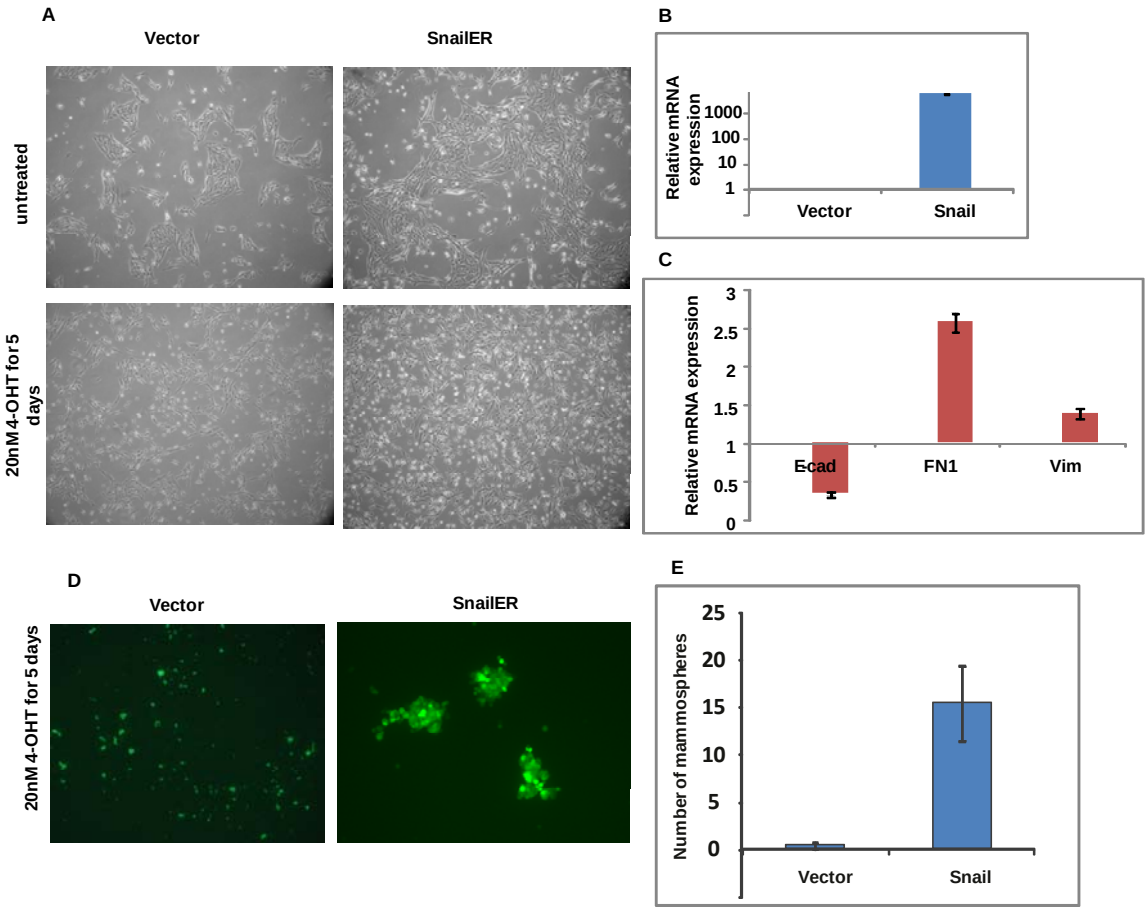


Figure S3. Induction of EMT promotes mammosphere formation in primary human mammary epithelial cells (HMECs) (**A**) Phase-contrast images of Snail-ER or control vector infected cells treated with tamoxifen for 5 days. (**B**). Expression of Snail mRNA in HMECs, which were induced to undergo EMT

by ectopic expression of Snail-ER, as determined by Real-time RT-PCR. Expression levels are reported relative to the levels in cells containing the control vector. GAPDH mRNA was used to normalize variability in template loading. The data are reported as mean +/- SEM (**C**). The expression levels of mRNAs associated with EMT in primary HMEC-Snail-ER cells induced to undergo an EMT by exposure to tamoxifen for five days. Expression levels are reported relative to the expression levels in control vector cells also treated with tamoxifen for 5 days. GAPDH mRNA was used to normalize the variability in template loading. The data are reported as mean +/- SEM. (**D**). Microscopic images of mammospheres seeded by either primary HMEC-Snail-ER (right) or control cells (left) treated with tamoxifen for 5 days. The lentiviral vectors used also carried a green fluorescent protein (GFP) marker gene. (**E**). Quantification of mammospheres formed by equal numbers of the cell types described in panel D. The data are reported as mean +/- SEM.

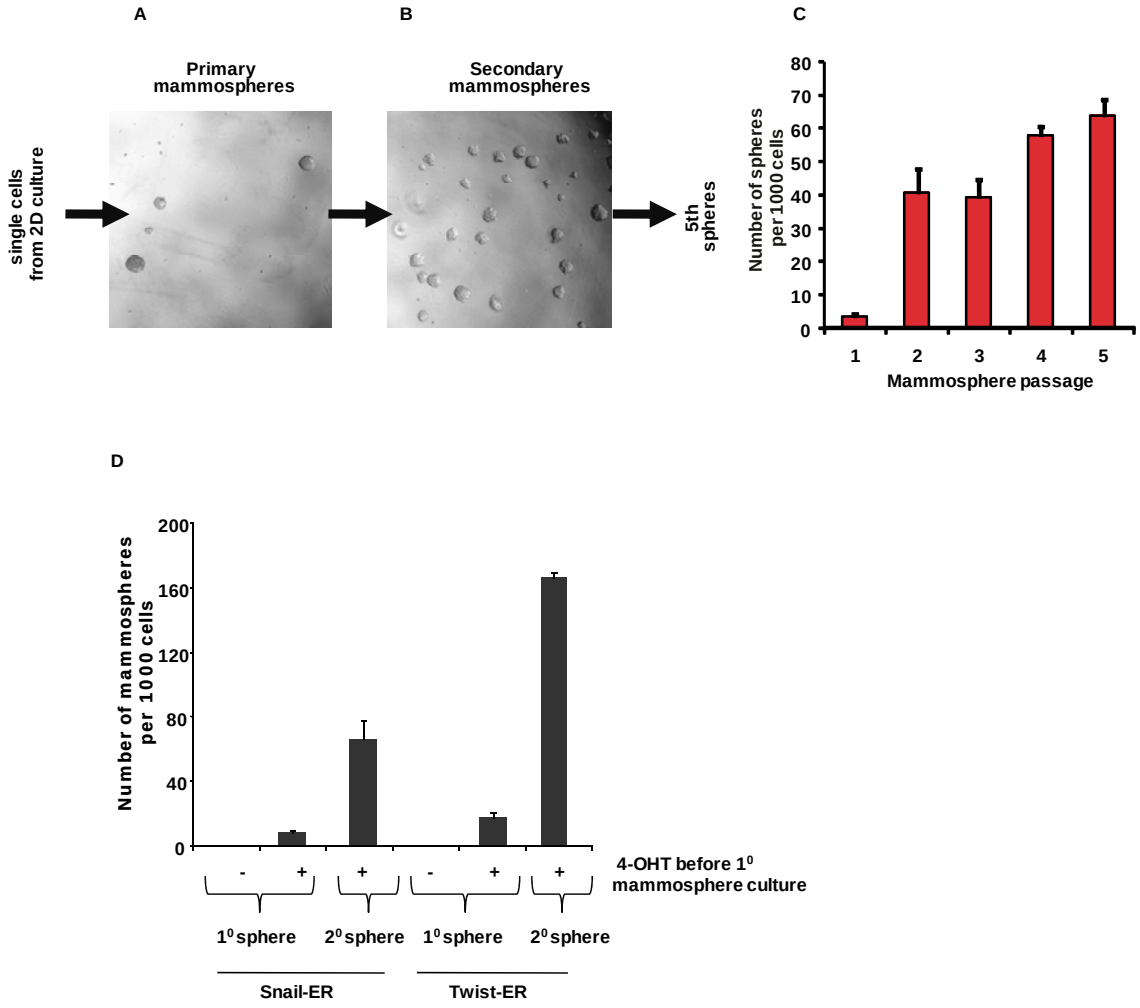


Figure S4. *In vitro* passaging of mammospheres increases the number of mammosphere-forming cells. (**A**). Primary mammospheres derived from 2D cultured cells. (**B**). Secondary mammospheres, each arising from a single cell extracted from a primary mammosphere. (**C**). Quantification of the mammospheres formed during 5 serial passages; on average, each mammosphere has about 300 cells and ~ 5% of these cells can re-initiate (i.e., seed) new mammospheres. This suggests that each mammosphere contains about 15 mammosphere-initiating cells and that during each mammosphere passage the mammosphere-initiating cells are expanded by ~15 fold. The data are reported as mean +/- SEM. (**D**). Quantification of primary and secondary mammospheres arising from HMLE-Snail-ER or Twist-ER cells treated with or without tamoxifen for 12 days in 2D culture and then cultured as mammospheres without continuous tamoxifen treatment. The primary mammospheres were dissociated into single cells and cultured for secondary mammospheres in the absence of tamoxifen.

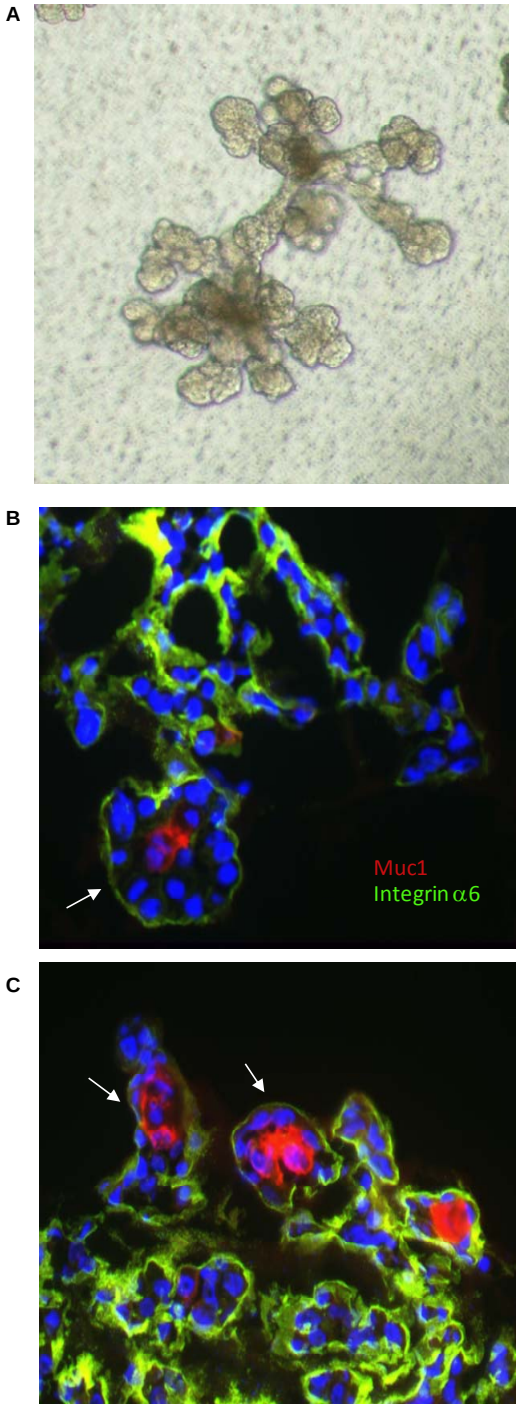


Figure S5. *In vitro* differentiation of mammospheres in Matrigel promotes the formation of secondary structures. (**A**). Phase-contrast images of the differentiated mammospheres following culture in Matrigel (**B & C**). The differentiated structures were immunostained for Muc1 (red) and CD49f/integrin α6 (green).

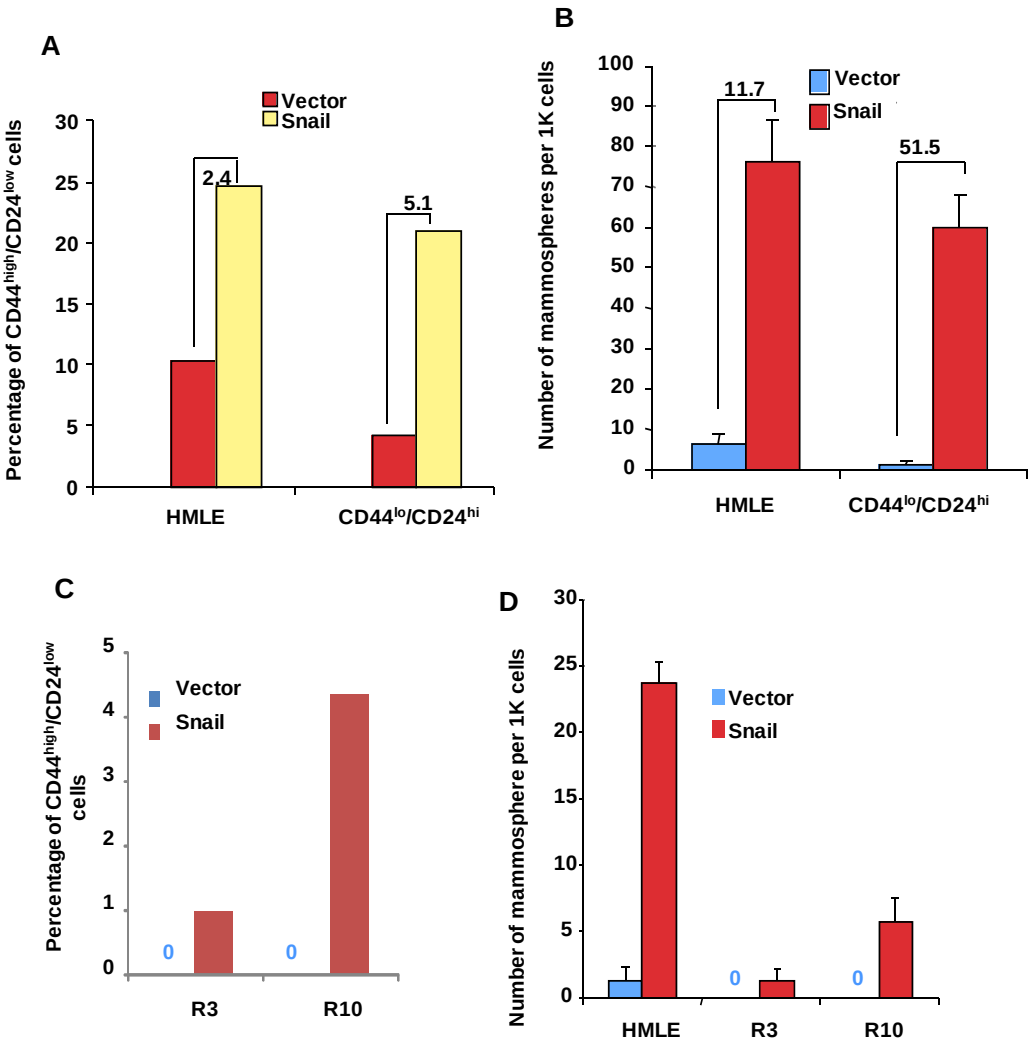


Figure S6. Induction of an EMT in either CD44^{low}/CD24^{high} cells or in single-cell clones derived from CD44^{low}/CD24^{high} cells promotes the formation of stem-like CD44^{high}/CD24^{low} cells. (**A**). HMLEs or CD44^{low}/CD24^{high} cells were infected with the Snail vector or a control vector. The relative increase in the HMLE CD44^{high}/CD24^{low} subpopulation measured 7 days after vector infection and ongoing drug selection is represented in the bar graph. (**B**). Graph shows numbers of mammospheres formed/1000 of Snail- or control vector-infected HMLEs or CD44^{low}/CD24^{high} populations. The fold change is indicated in the graph. (**C**). Two single-cell clones (R3 and R10) derived from CD44^{low}/CD24^{high} populations were infected with Snail or control vector, and the percentage of CD44^{high}/CD24^{low} population is represented as bar graph. (**D**). Numbers of mammospheres generated by 1,000 Snail- or control vector-infected R3 and R10 single-cell clones are represented in this bar graph; parental HMLE cells infected with the Snail vector *(left)* were used as a control for the mammosphere assay.

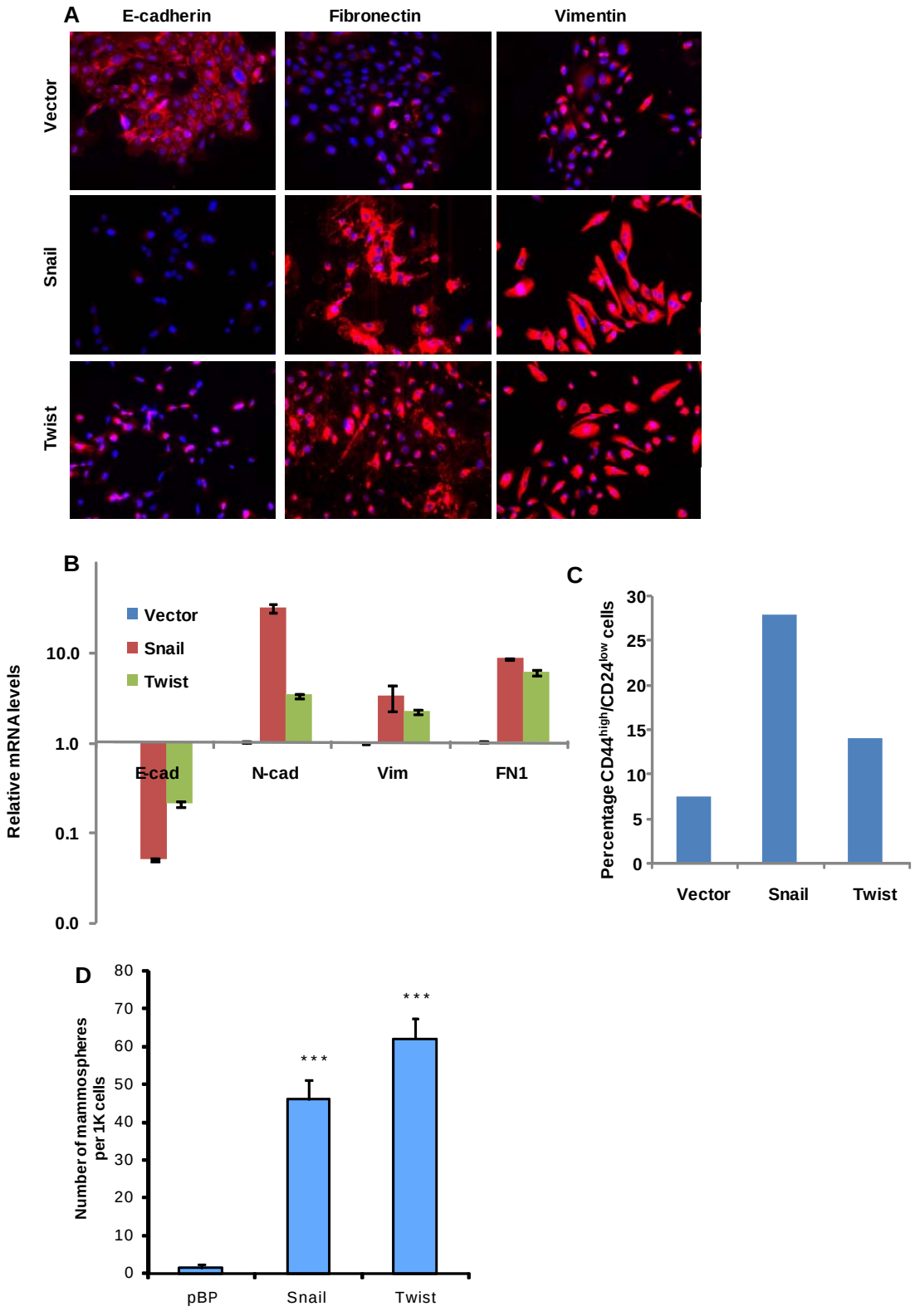


Figure S7. Induction of an EMT in HMLER cells using Snail, Twist, or a control vector (**A**). Immunostaining of HMLER cells induced to undergo EMT

by ectopic expression of Snail or Twist, using antibodies against E-Cadherin, N-cadherin, fibronectin and vimentin. Immunostaining of control cells is shown for comparison (**B**). Expression of mRNAs associated with EMT in HMLERs that were induced to undergo EMT by the ectopic expression of Snail or Twist relative to control vector-infected cells, as determined by Real-time RT-PCR. GAPDH mRNA was used to normalize variability in template loading. The data are reported as mean +/- SEM. (**C**). The percentage of $CD44^{high}/CD24^{low}$ in cells in HMLER cells that underwent an EMT induced by ectopic expression of Snail, Twist or control vector. (**D**). Number of mammosphere/1000 HMLER cells expressing Snail, Twist or control vector. The data are reported as mean +/- SEM.

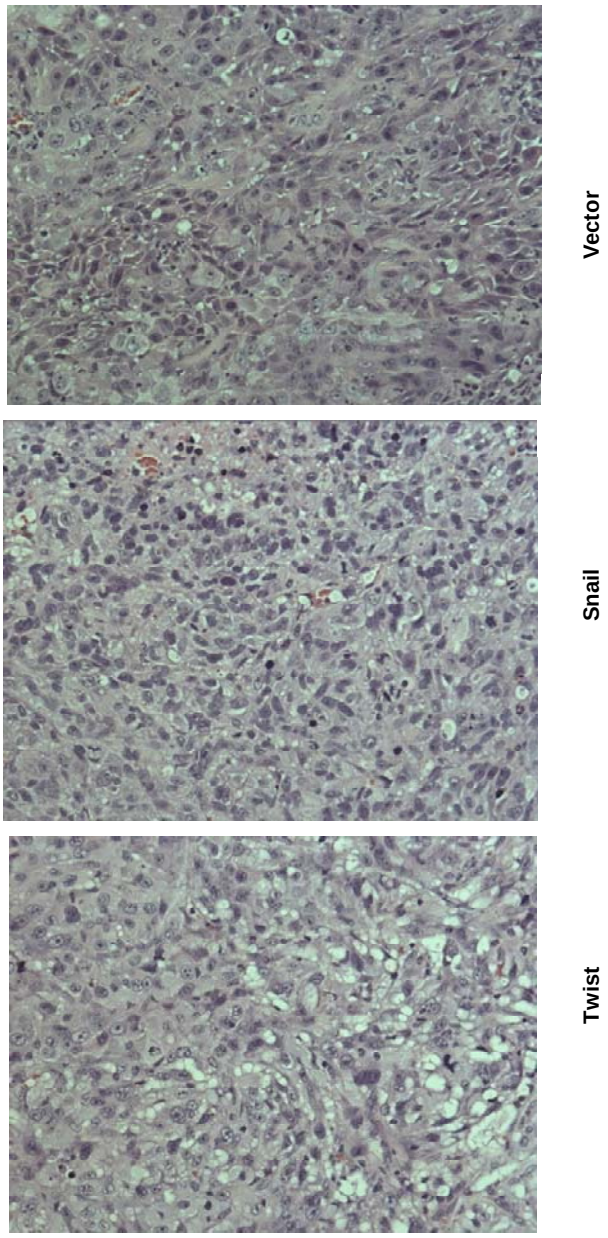


Figure S8. H&E staining of tumors derived from HMLER cells expressing Snail, Twist, or the control vector. Light microscope images (10X) of the tumors derived from (**A**). Vector (**B**). Snail (**C**). Twist expressing HMLER cells.

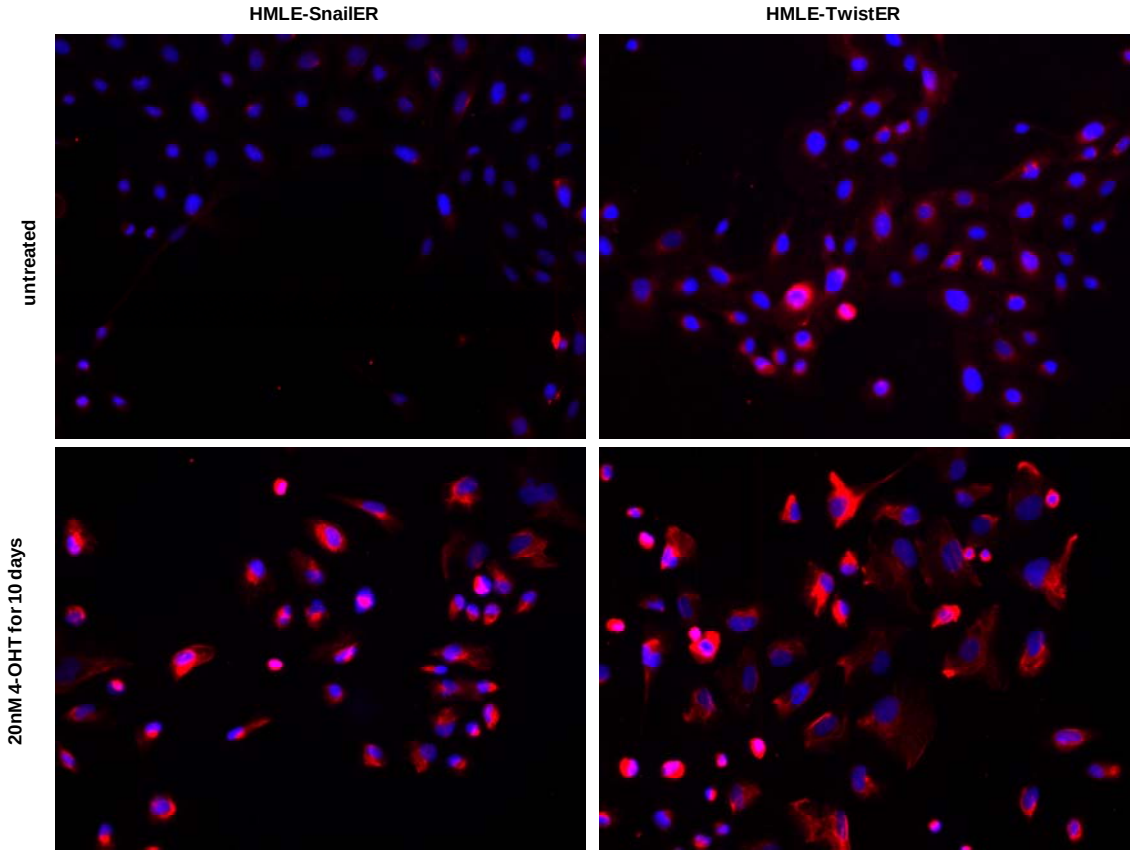


Figure S9. Vimentin immunostaining of HMLE-Snail-ER or Twist-ER cells that underwent an EMT in response to treatment of tamoxifen for 12 days.

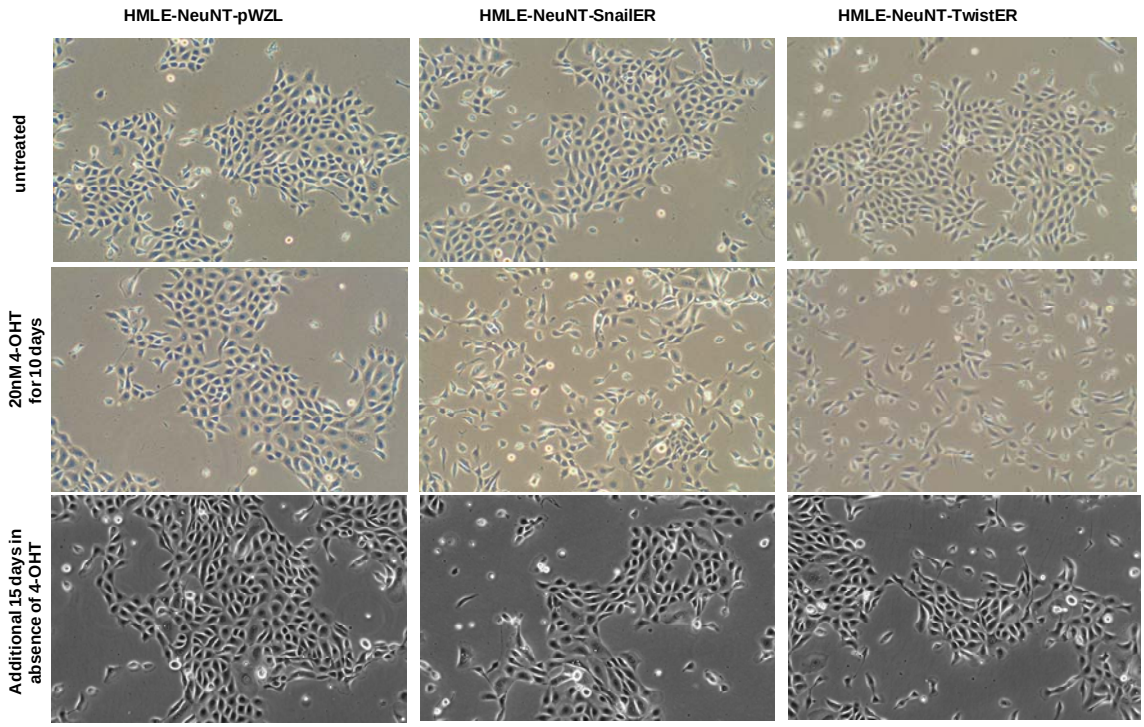


Figure S10. Phase contrast images of HMLEN-Snail-ER or HMLEN-Twist-ER cells that underwent EMT after ten days of tamoxifen (4-OHT) treatment (middle row) or were left untreated (top row). Following this 4-OHT treatment, 4-OHT was withdrawn and cells were observed 15 days later. Uninfected cells or cells infected with the indicated viral vectors are shown in the columns.