CD49f and CD61 identify Her2/neu-induced mammary tumor initiating cells that are potentially derived from luminal progenitors and maintained by the integrin-TGFβ signaling

Supplementary Materials and Methods

Cell isolation

Lin$^-$ epithelial cells from mammary glands were isolated using the EasySep negative selection kit (StemCell Technologies, Vancouver, BC, Canada) according to the manufacturer's instructions. In brief, mammary glands were minced and incubated in digestion medium (300U/mL collagenase, 100U/mL hyaluronidase, 5% FBS, 10ng/mL EGF, 10 ng/mL FGF, 4μg/mL heparin in EpiCult-B basal medium) for 5h at 37^{o}C with constant agitation. Lin$^-$ epithelial cells were enriched by removing CD45$^+$/Ter119$^+$, CD31$^+$ and CD140a$^+$ cells using antibodies against those respective surface antigens and the EasySep magnet. Isolated cells were kept on ice until analysis.

Quantitative real-time RT-PCR analysis

Total RNA isolated from H6O5 cells was reverse-transcribed using M-MLV reverse transcriptase (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. We carried out quantitative real-time RT-PCR using RT^2 Fast SYBR® Green/ROX™ qPCR Master Mix (SABiosciences, Frederick, MD, USA) and primers indicated in Supplementary Table 1. Data analysis was performed using the $2^{-\Delta\Delta C}T$ method for relative quantification, and all sample values were normalized to the glyceraldehyde-3-phosphate dehydrogenase (Gapdh) expression value (as the internal reference control).

Cell Proliferation Analysis

1×10^4 cells were plated in 24 well plates with 500μl complete medium. Next day old medium was replaced with fresh medium containing SB431542 or A-83-01 at the concentration as indicated and cells were further cultured. Cell proliferation assays were performed every day up to a period of 5 days. At the desired time points, 50μl of the 5

mg/ml MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) (Sigma, St. Louis, MO, USA) solution was added to each well. Cells were incubated at 37°C in a CO_2 incubator for 4 hr, and formazan crystals formed in each well during the incubation period were dissolved in 500µl of Ethanol: DMSO (1:1) mixture. 100µL of the dissolved crystals were transferred to a 96 well plate and the optical density was read on an ELISA plate reader at 595 nm against a reference wavelength of 630 nm. Growth curve was prepared by plotting OD at 595 nm (on *y* axis) against time (on *x* axis).

Western blot analysis

Protein concentration was measured using BCA kit (Thermo Scientific, Rockford, IL, USA) and protein lysates were separated by SDS-PAGE and then blotted onto Hybond-C extra membranes (GE Healthcare, Buckinghamshire, UK) for Western blot analysis. Antigen-antibody reaction was detected using Super Signal West Pico and Femto chemiluminescent substrate kits according to manufacturer's instructions (Thermo Scientific). The primary antibodies used for Western blot analysis are phosphor-FAK (Tyr397), FAK, phosphor-c-Src (Tyr416), c-Src, phospho-Smad2 (Ser465/467), Smad2, phospho-Smad3 (Ser423/425), Smad3 (Cell Signaling Technology, Beverly, MA), actin (c4/actin, Becton Dickinson) and Neu (C-18, Santa Cruz Biotechnology, Santa Cruz, CA).

siRNA transfection

siRNA transfections were performed with 20 nM of each siRNA using OligofectamineTM RNAiMAX (Invitrogen) according to the instructions of the manufacturer. The mouse CD61 siRNA (siGENOME SMARTpool) and control siRNA were purchased from Dharmacon (Boulder, CO).

Statistical analysis

The frequency of TICs in the total cell population was calculated based on a Poisson probability distribution as described previously (Liu *et al.*, 2007). The Student's t-test was used to analyze the significance of difference between two groups of data. $P < 0.05$ was regarded as statistically significant.

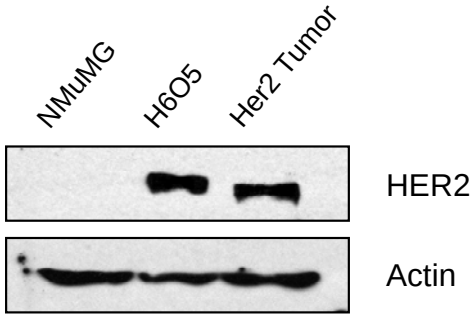
Supplementary Table 1 Primer sequences for real-time RT-PCR analysis

Gene Name	Forward Primer	Reverse Primer
Abcg2	TTCACTTGTATATTATACTTCATGTTAGGACTG	CGATTGTCATGAGAAGTGTTGCT
Aldh1	GCTGGGGTGGTGTGGGTT	GACCATGTTCACCCAGTTCTCTTC
Bmi1	GAGAAGCCTAAGGAAGAGGTGAATG	CAGGTATAAATGTAGGCAATGTCCA
CD133	TTATATGGTGTTCACAATCCTGTTATGAC	GCTTTCCATGTTGACTATCTTGTTGTTC
CD29	CAAGTGGGACACGGGTGAA	CTACTGTGACTAAGATGCTGCTGCTG
CD44	GCGGTCAATAGTAGGAGAAGGTGTG	GCACCATTTCCTGAGACTTGCTG
CD49f	GGCTCTATTAGTGTTTTTACTGTGGAAGTG	AATACTATGCATCGGAAGTAAGCCTCTC
CD61	GAGCCAAGTGGGACACAGCA	CGTCATCTGAAGATGGTCTCATTAAGT
ESA	ATGAGAAGGCTGAGATAAAGGAGATG	AGTCCGAGCTCTTCTGCCACT
Gli1	TCGGACCCACTCCAATGAGAA	CTCGATGCCGCTTGGTCAC
Gli2	GCTTGGACTGACACAGGAGCA	AGTGGCAGTTGGTCTCGTAGATG
Jag1	GATGGGAACCCTGTCAAGGA	GAGGAACCAGGAAATCTGTTCTGT
Mdr1	TCATTTGCTCCTGACTATGCGA	TTTACATTTCCTTCTAACAGAGTAGGCTTC
Nanog	GAACTCTCCTCCATTCTGAACCTGA	TGGTGCTGAGCCCTTCTGAATC
Notch1	ATGTCAATGTTCGAGGACCAGATG	GATGAAGTCAGAGATGACAGCAGGTG
Notch2	AACAGAGATATGCAGGACAATAAGGAAG	GCGGTCCATGTGGTCAGTG
Notch3	CAGTGGATGAGCTTGGGAAATCT	ACAGCGGCGTCTCTTCCTTG
Oct3/4	CGGAAGAGAAAGCGAACTAGCA	TACAGAACCATACTCGAACCACATC
Procr	AGACTGCCGTGTGGGTGTCA	CGACCTGTTTGGCTCCCTTTC
Sca1	GCCCTACTGTGTGCAGAAAGAG	CTTTACTTTCCTTGTTTGAGAATCCA
Sox1	GAAAACCCCAAGATGCACAACTC	GCTTCTCGGCCTCGGACA
Sox2	CTTCGCAGGGAGTTCGCA	CAAATTCTCAGCTTATAAACAATGGACA
Sox4	GGCTGGGGACTCGAAGGA	GGAAGCTCGTTGGAAGGGTG
Stat3	GGAAAAGGACATCAGTGGCAAGA	GGGTAGAGGTAGACAAGTGGAGACA
Tgfbr2	CAATGCTGTGGGAGAAGTGAAG	CTCGGTCTCTCAGCACACTGTC
Tp63	ACAGACTGCAGCATTGTCAGTTTC	CTTCAGACTTGCCAAATCATCCA
CD24	GCTCCTACCCACGCAGATTTACT	CCCCTCTGGTGGTAGCGTTACTT
Krt5	TGACAACGTCAAGAAGCAGTGTG	TCCTGGTACTCCCGCAGCA
Krt6	GATCGACCACGTTAAGAAGCAGTGT	GCATCCTCCAGCCCTTCCAG
Krt14	GAGGAATGGTTCTTCAGCAAGACA	GGTTATTCTCCAGGGATGCTTTCA
Krt18	TGGACAAGTACTGGTCTCAGCAGATT	CTGTTCTCCAAGTTGATGTTCTGGTT
Pai	AGGTAAACGAGAGCGGCACAGT	GCCCATGAAGAGGATTGTCTCTGTC
Il6	AAATCGTGGAAATGAGAAAAGAGTTGT	TTTCCTGATTATATCCAGTTTGGTAGCA
Igfbp3	GAAGGGGTTCTATAAGAAGAAGCAGTG	CACGCTGAGGCAATGTACGTC
Cdh2	GCTGATCCTTGTTCTCATGTTTGTG	GCTGGCTCAAGTCATAGTCCTGGT
Foxc2	GTGTCCACTGGATAAGGTTTCGTCT	CATCTACTGTACAAAGCCATGCACTTC
Sma	CGTGAGATTGTCCGTGACATCAAG	CCCGCTGACTCCATCCCA
Snail	GCCTTGTGTCTGCACGACCTG	TCTTCACATCCGAGTGGGTTTG
Twist1	CCACGAGCGGCTCAGCTAC	CCTTCTCTGGAAACAATGACATCTAGG
Zeb1	AGACACAAATATGAGCACACAGGTAAGA	GCTTGCCACACTTGTCACATTG
Gapdh	GTCGGTGTGAACGGATTTGG	CGTGAGTGGAGTCATACTGGAACA

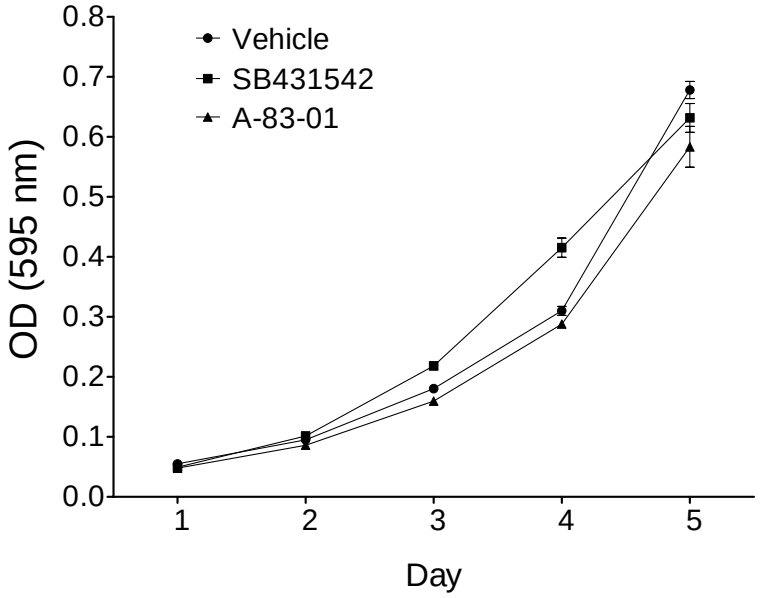
Supplementary Table 2 Expression profiling analysis of stem cell signature and differentiation marker genes in H6O5 cells vs. NMuMG cells

Gene Classification	Full Gene Name	Gene Symbol	Expression fold (H6O5/NMuMG)
Stem cell signature genes	ATP-binding cassette, sub-family G (WHITE), member 2	Abcg2	15.267
	aldehyde dehydrogenase 1 family, member A1	Aldh1 (Aldh1a1)	0.001
	Bmi1 polycomb ring finger oncogene	Bmi1	1.542
	hematopoietic stem cell antigen (prominin-1)	CD133 (Prom1)	1.576
	antigen CD29 includes MDF2 (integrin, beta 1)	CD29 (Itg1)	1.649
	cell surface glycoprotein CD44	CD44	0.459
	CD49 antigen-like family member F (integrin, alpha 6)	CD49f (Itga6)	3.741
	antigen CD61 (integrin, beta 3)	CD61 (Itgb3)	8.081
	epithelial cell adhesion molecule	ESA (Epcam)	467.431
	GLI-Kruppel family member GLI1	Gli1	1.009
	GLI-Kruppel family member GLI2	Gli2	0.036
	Protein jagged-1 (antigen CD339)	Jag1	0.297
	ATP-binding cassette, sub-family B (MDR/TAP), member 1	Mdr1 (Abcb1)	0.001
	homeobox transcription factor Nanog	Nanog	39.266
	transmembrane receptor Notch1	Notch1	1.652
	transmembrane receptor Notch2	Notch2	0.325
	transmembrane receptor Notch3	Notch3	161.781
	POU domain, class 5, transcription factor 1	Oct3/4 (Pou5f1)	6.502
	protein C receptor, endothelial	Procr	0.089
	lymphocyte antigen 6 complex, locus A	Sca1	1.68
	SRY (sex determining region Y)-box 1 transcription factor	Sox1	0.121
	SRY (sex determining region Y)-box 2 transcription factor	Sox2	0.002
	SRY (sex determining region Y)-box 4 transcription factor	Sox4	0.897
	signal transducer and activator of transcription 3	Stat3	2.471
	transforming growth factor, beta receptor II	Tgfbr2	1.355
	tumor protein p63	Tp63	116.995
Differentiation marker genes	CD24 antigen (small cell lung carcinoma cluster 4 antigen)	CD24	0.315
	cytokeratin-5	Krt5	5.215
	cytokeratin-6	Krt6	1.147
	cytokeratin-14	Krt14	1783.921
	cytokeratin-18	Krt18	0.565

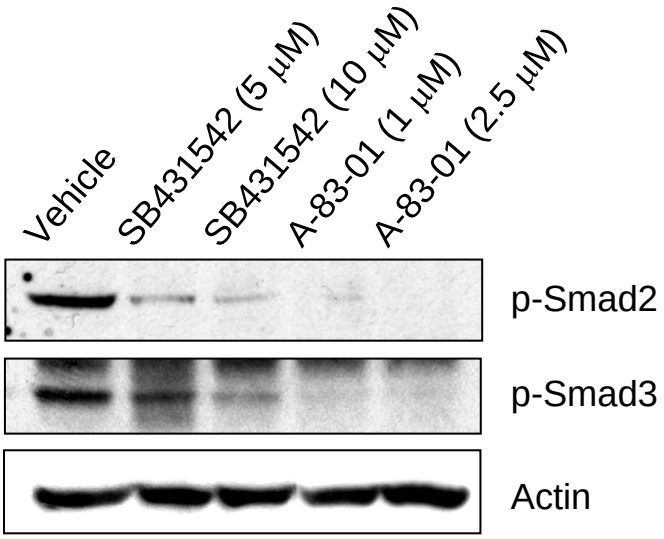
Supplementary Figures



Supplementary Figure 1 HER2 protein expression in NMuMG, H6O5 cells and primary Her2 tumor. Actin protein expression was analyzed to show equal loading of protein lysate.



Supplementary Figure 2 Proliferation analysis of TGFβ inhibitor-treated H6O5 cells. MTT assay was performed to measure the proliferation of H6O5 cells during five-day treatment with TGFβ inhibitors. H6O5 cells were treated with 10 μM SB431542 or with 1 μM A-83-01.



Supplementary Figure 3 Western blot analysis of phosphorylation status of TGFβ-downstream-signaling mediators in TGFβ inhibitor-treated H6O5 cells. Phosphorylation of Smad2 (Ser465/467) and Smad3 (Ser423/425) was examined by using anti-phospho-Smad2 and anti-Smad3 antibodies. Actin protein expression was shown as a loading control.