Supporting Information

Zucchi *et al.* 10.1073/pnas.0808978105

SI Experimental Procedures

Mammosphere Culture. Mammosphere culture was performed as described by Dontu *et al.* (1). For generation of mammospheres, LA7 cells were grown in low attachment plates (Bibby Sterilin) at clonogenic density (1) or at the dilution of one cell per well in 96-well plates for 7–10 days (2). For testing long-term mammosphere-regeneration capability, each single-cell–generated mammosphere was mechanically and enzymatically dissociated with 0.05% trypsin and 0.53 mM EDTA (Invitrogen), and the cells were plated again at a density of one cell per well. Efficiency of single-cell dissociation was monitored microscopically. Mammospheres were microscopically monitored and counted.

Cell Transplantation in NOD-SCID Mice. For each condition, a minimum of six fat pads were used. The size of the tumors was measured through the animal's skin; initial measurements were performed immediately after injection and continued every other day until day 80 or less. The animals were killed at day 90 (or earlier). Tumors for the third, fourth, and fifth generations obtained by LA7SL were measured once at day 80 after cell injection. Tumors that showed growth greater than 1.5 mm^3 were considered palpable tumors. The tumors removed were used to isolate RNA or genomic DNA or were fixed in formalin and embedded in paraffin for further analysis or to establish cell lines (2).

Mammary Tumor Dissociation; Isolation of LA7SL, LA7E, and LA7ML Cells; and Single-Cell Subcloning. Tumors were mechanically and enzymatically dissected, as described (2). Cells were plated at low density to ensure that each colony was derived from a single cell. Colonies were then microscopically inspected for their morphology; 20–40 colonies were collected for each cell type. Cell type-specific isolation was made possible by using the cloning ring method. Subcloning of LA7SL cells or LA7E was performed as described for LA73F12ms (2). Cells from all colonies were tested for their capacity to generate serially and sustain sphere and tubule formation *in vitro* and for trilineage differentiation potential, by plating single-cell–generated spheres (derived from cell lines established from each colony) onto collagen and by testing the resulting outgrowths for expression of K18, K14, and β-casein (not shown), as described (2). Sphere and tubule sustainability was assessed as follows: first passage of a single sLA7 cell–derived sphere and tubule, obtained at 7–14 days, was dissociated into single-cell suspensions and used to regenerate new spheres and new tubules, respectively, at 7–14-day intervals (2).

Immunohistochemistry. The following primary mouse monoclonal antibodies were used: from Santa Cruz Biotechnology: anti-p21 (SC6246), anti-p63 (SC8431), and anti-CD44/HCAM (SC9960); from Novocastra: anti-E-Cadherin (36B5); from Sigma: anti-K18 (clone K8.13) and anti-αSMAI (A2547); and from NeoMarkers: anti-Ki67 (SP6), anti-K14 (LL002), and anti-vimentin (V9). Secondary antibodies were all from Santa Cruz Biotechnology: rabbit anti-goat, goat anti-mouse, and goat anti-rabbit antibodies.

Mammosphere Immunohistochemistry. Mammosphere were cryoembedded in OCT compound (Sakura Tissue-Tek), and sections of 8-μm thickness were generated. Sections were stained with antibodies against p63 (Santa Cruz Biotechnology).

Flow Cytometry. Single-cell suspensions were washed in PBS and 3% FCS and were stained with phycoerythrin-labeled anti-rat CD44 monoclonal antibody (SC53068Clone OX49; Santa Cruz Biotechnology) or phycoerythrin-labeled isotope control antibody (Santa Cruz Biotechnology) using 1 μg of antibody per 10^6 cells in a volume of 100 μl. After 20 min on ice, cells were washed again and resuspended in PBS and 3% FCS supplemented with 1 μg/ml DAPI to allow exclusion of nonviable cells. Flow cytometry was performed using a FACSAria cell sorter (BD Biosciences), and data were analyzed using FlowJo software (Tree Star).

Genomic DNA and RNA Isolation and PCR Analysis. RNA and genomic DNA were isolated as described (2); PCR analysis was used to confirm in LA7SL, LA7E, or LA7ML cultures the lack of mouse DNA and presence of rat DNA. PCR analysis was performed using the following primers—forward: 5′-CCACAACACCTTACCTATCTC-3′ and reverse: 5′-CTCCCCCTTCCTTTATAAC-3′, identical for mus musculus cDNA or rat cDNA, designed on the sequence AK008780_m L.S.

Western and Northern Blot Analysis of sLA7/LA7SL, LA7E1, and LA7E3 Cells. To distinguish sLA7 from LA7SL, LA7E1, LA7E2, and LA7E3 cells, cells were cultured to obtain sufficient numbers for Northern and Western blot analysis as in the study by Zucchi *et al.* (2). Blots were probed with a cDNA probe amplified with primers—forward: 5′-TTCACCCAGCTCAATCTC-3′ and reverse: 5′TACATCACACCTCCTCCTC-3′), designed from the CD44 mRNA sequence ac, NM_009851.

Antisense Oligonucleotide Methodology. For the inhibition study of p63 protein expression, three antisense oligonucleotides, each containing 21 bases, were synthesized from the rat sequence NM_019221 of *p63* mRNA: AS-p63/1 (5′AGAGTAACGATGATTAAAATT3′, spanning nucleotides 1107–87), AS-p63/2 (5′AAGCAAGAGTATTGAAGGCTT3′, spanning nucleotides 2382–2362), and AS-p63/3 (5′AAGTTAATGATGCTTATCATT3′, spanning nucleotides 4254–4234). Three sense oligomers complementary to the three antisense oligomers were also used. Oligos were added to the cells at a concentration of 50 μg/100 μL of tissue culture media with 2 administrations per day. After 96 h, the oligos were removed and the cells were inspected for mammosphere generation. Sphere formation was observed from day 7 to day 10. Mammospheres were then trypsinized, and dissociated cells were replated to regenerate mammospheres.

Cell Proliferation Measurements by 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyl Tetrazolium Bromide (MTT). Cell proliferation was carried out using the MTT kit, as suggested by the manufacturer (Roche Applied Science).

1. Dontu G, Liu S, Wicha MS (2005) Stem cells in mammary development and carcinogenesis: Implications for prevention and treatment. *Stem Cell Review* 1:207–213.

2. Zucchi I, *et al.* (2007) The properties of a mammary gland cancer stem cell. *Proc Natl Acad Sci USA* 104:10476–10481.

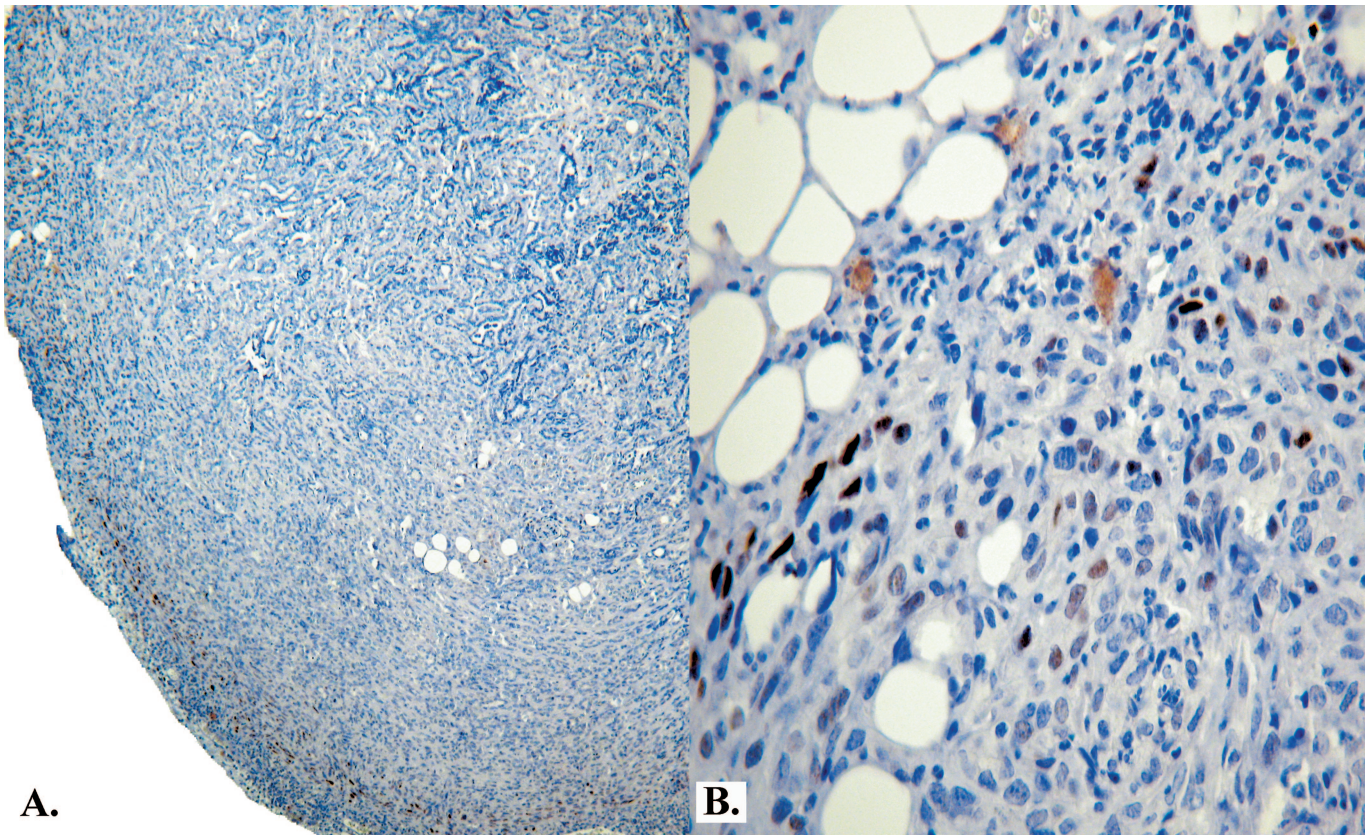

Fig. S1. p21/^{WAF1} staining of a tumor generated by 10^5 sLA7 cells. (Magnification: *A*, ×5; *B*, ×40.)

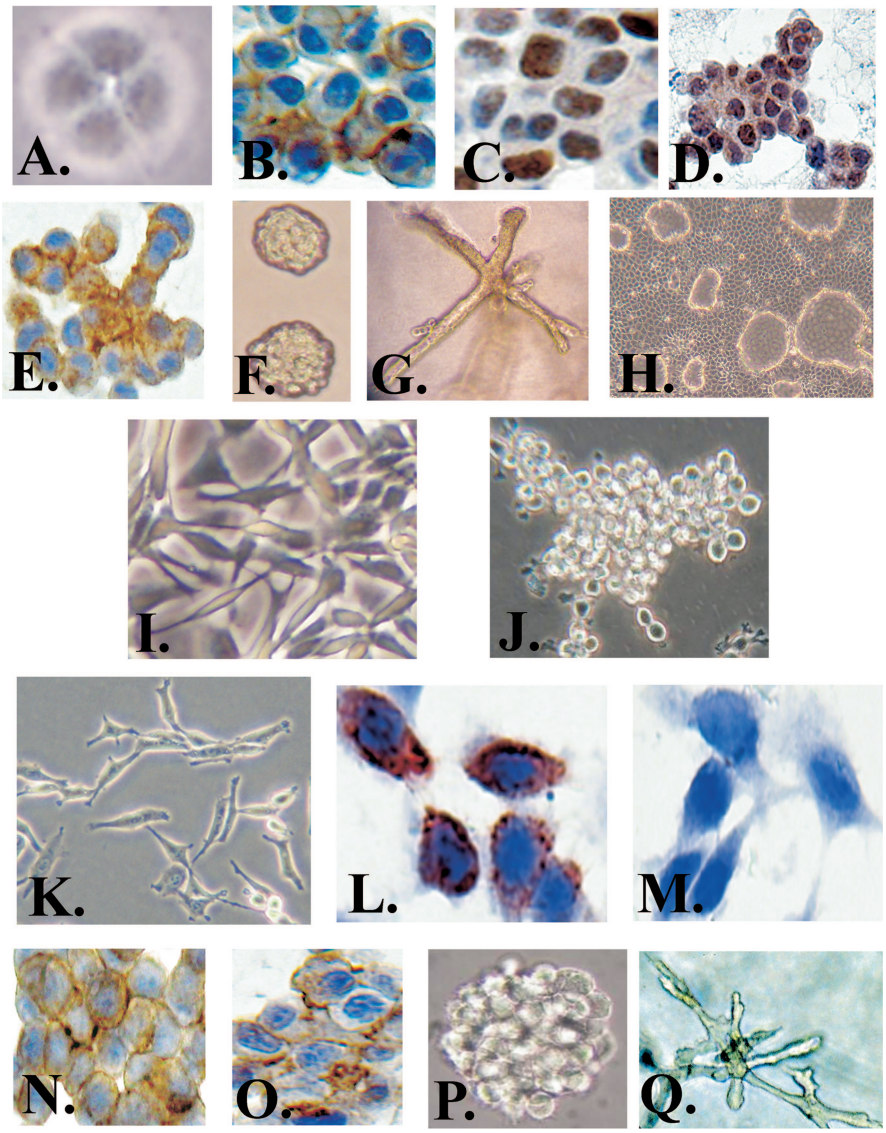

Fig. S2. Characterization of different cell types isolated from tumors generated by 10^5 sLA7 cells. LA7SL cell analysis: LA7SL holoclone 16 (*A*) and antibody staining for E-Cadherin (membrane, brown) (*B*), p21/^{WAF1} (nuclear, brown) and K14 (negative) (*C*), p63 (nuclear, brown) (*D*), and CD44 (membrane, brown) (*E*). Blue staining is for Hoechst nuclear dye 33342. Single LA7SL cell generates mammospheres (*F*), tubuli (*G*), and domes (*H*). LA7ML cell analysis: LA7ML cells (*I*) and disorganized cell aggregate generated by a LA7ML cell (*J*). (*K*) LA7E cell analysis: LA7E1 clone 12. Antibody staining for p21/^{WAF1} (negative) and K14 (cytoplasm, red) (*L*), p21/^{WAF1} (negative) and K18 (negative) (*M*), CD44 (membrane, brown) (*N*), and E-Cadherin (membrane, brown) (*O*). Blue staining is for Hoechst nuclear dye 33342. LA7E1 clone 12 cells (passage 2) generate mammospheres (*P*) and tubuli (*Q*). All cell types were confirmed as rat cells by testing with rat-specific PCR primers. (Magnification: *H*, ×10; *D*, *F*, *G*, *J*, *K*, *P*, *Q*, ×20; *B*, *C*, *E*, *I*, *N*, *O*, ×32; *A*, *L*, *M*, ×40.)

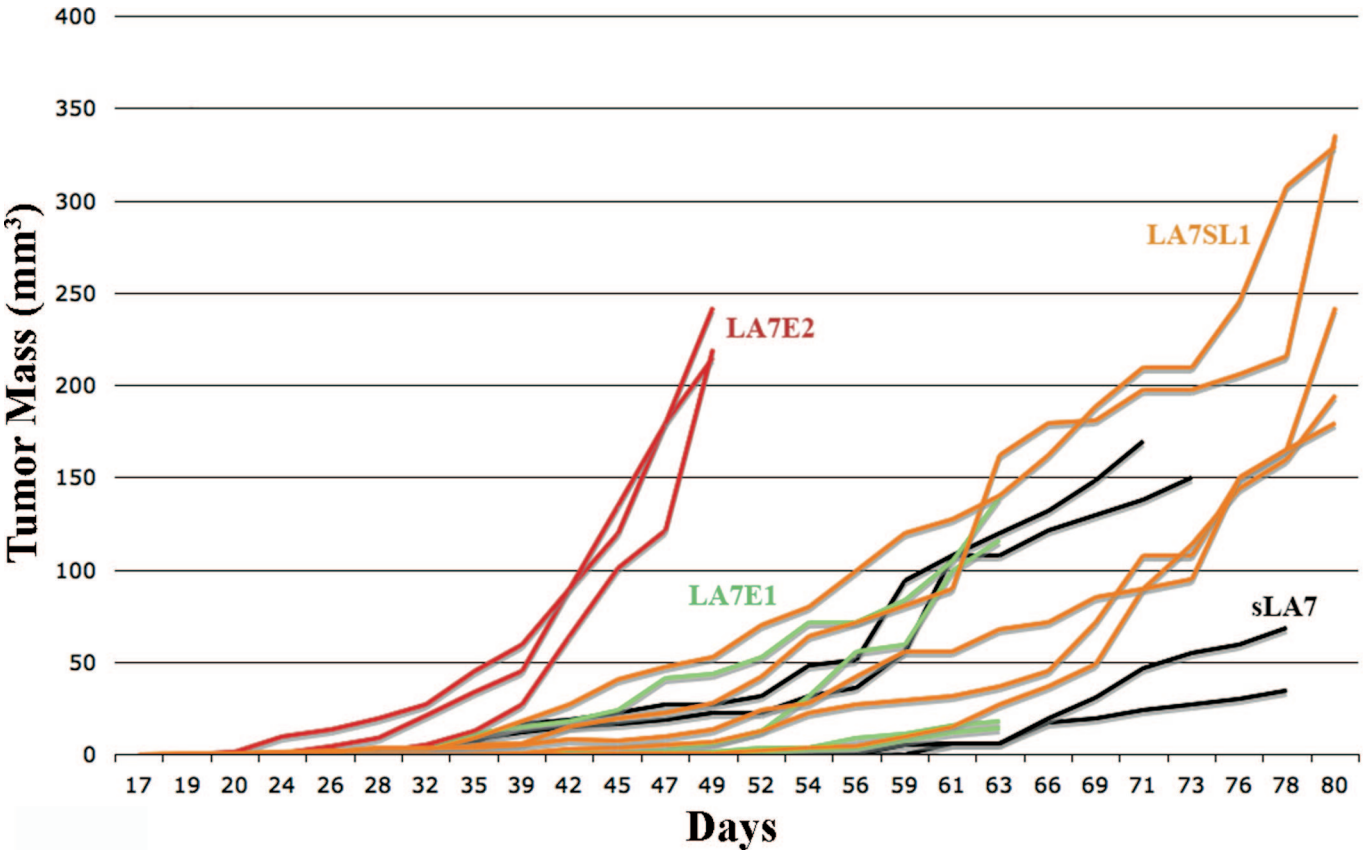

Fig. S3. Tumor growth curves obtained from injection of 100 sLA7, 100 polygonal (LA7SL), and 100 elongated epithelial (LA7E) cells in NOD-SCID mice. Tumor growth curves were generated by injection of sLA7, LA7SL1, LA7E1, and LA7E2 cells. Vertical axis: tumor mass in cubic millimeters. Horizontal axis: days at time of tumor measurement (from the day of injection).

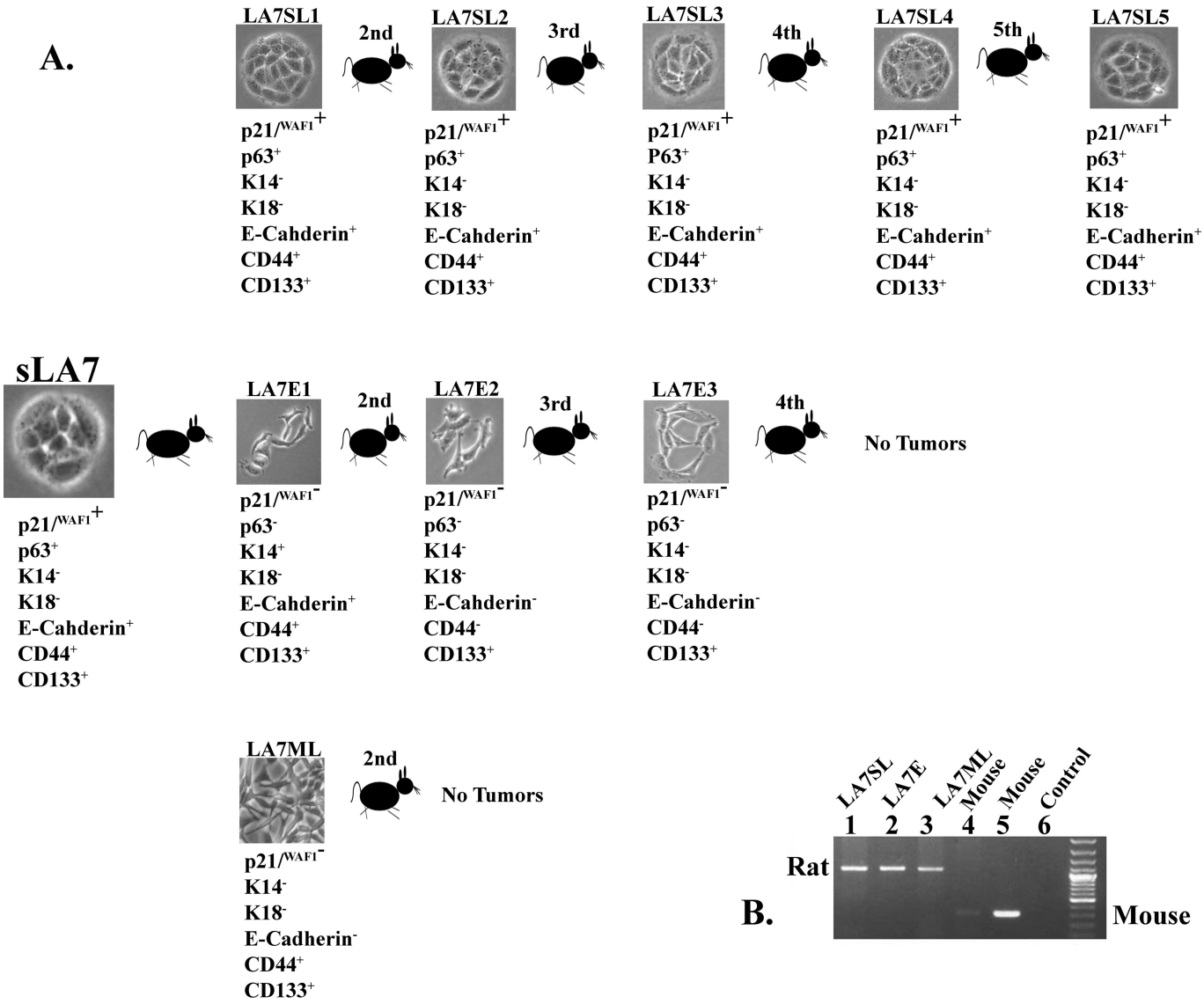

Fig. S4. Serial transplantation. (*A*) Scheme for serial transplantation with selected markers. (*B*) LA7SL, LA7E, and LA7ML cells are free of mouse cells. A unique pair of primers that amplify different sized fragments for mouse (300 bp) and rat (1100 bp) genomic DNA was used to confirm by PCR that the LA7SL, LA7E, and LA7ML cells were rat cells free of mouse cells. Lanes: LA7SL (1); LA7E (2); LA7ML (3); mouse genomic DNA, 20 ng (4); mouse genomic DNA, 100 ng (5); and no template as control (6).

Characteristics of Tumors generated from:

	Tumor Volume*	Total # Cells x10^8	Total # Cell Divisons
sLA7	35	0.35	18
	69	0.69	19
	150	1.5	20
	170	1.7	20
LA7SL1	180	1.8	20
	195	1.9	20
	242	2.4	21
	330	3.3	21
	338	3.4	21
LA7SL2	36	0.36	18
	84	0.84	19
	87	0.87	19
	240	2.4	21
	350	3.5	21
LA7SL3	25	0.25	18
	90	0.90	19
	180	1.8	20
	306	3.1	21
LA7SL4	31	0.31	18
	78	0.78	19
	110	1.1	20
	340	3.4	21
	301	3.1	21
LA7E1	21	0.21	18
	28	0.28	18
	122	1.2	20
	143	1.4	20
LA7E2	215	2.1	21
	220	2.2	21
	220	2.2	21

*Volume (in mm^3) at day of animal sacrifice

Fig. S5. Characterization of tumors generated by LA7SL, LA7E, and LA7ML cells. The tumor volumes were approximated using a spherical model based on the measurement of the three radii of the tumor. The number of cells of each tumor was estimated under the common approximation that 1 cm^3 of tumor contains 10^9 cells. The maximum passage number was estimated from the final number of cells of the tumor (at the time the animals were killed), taking into account that the initial number of cells injected was 100 and that all the cells duplicate [mathematically, total number of cell division = $\log_2$ (final number of cells/initial number of cells)] in each passage. The number of cell divisions is an estimation of the maximum number of cell divisions that each transplanted cell type may have undergone *in vivo* at the time the mice are killed.

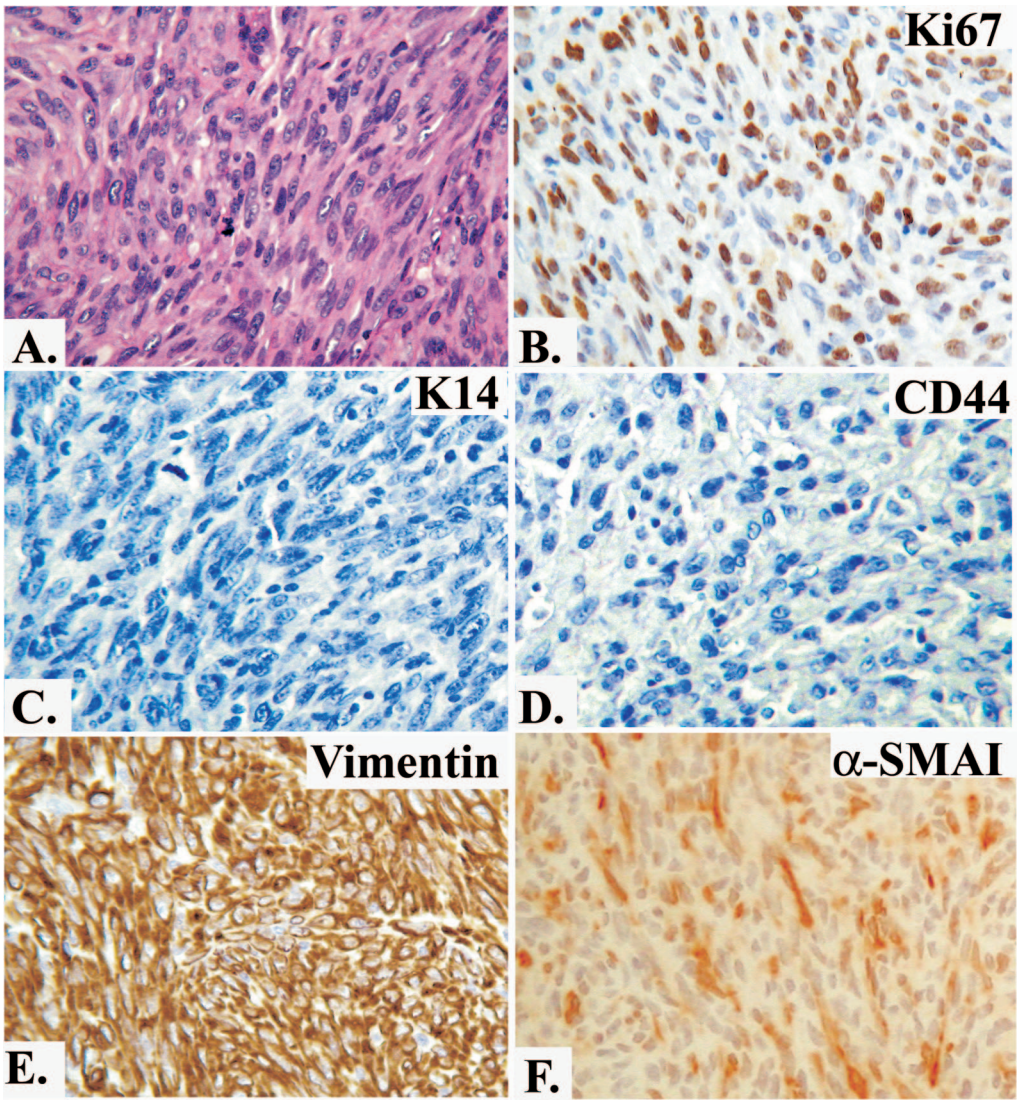

Fig. S6. Staining of a tumor generated LA7E3 cells. (*A*) H&E, core. Antibody staining for: Ki67, core (*B*); K14, core (*C*); CD44, periphery (*D*); vimentin, periphery (*E*); and α-SMAI, periphery (*F*). (Magnification: *B–F*, $\times$10; *A*, $\times$20.)

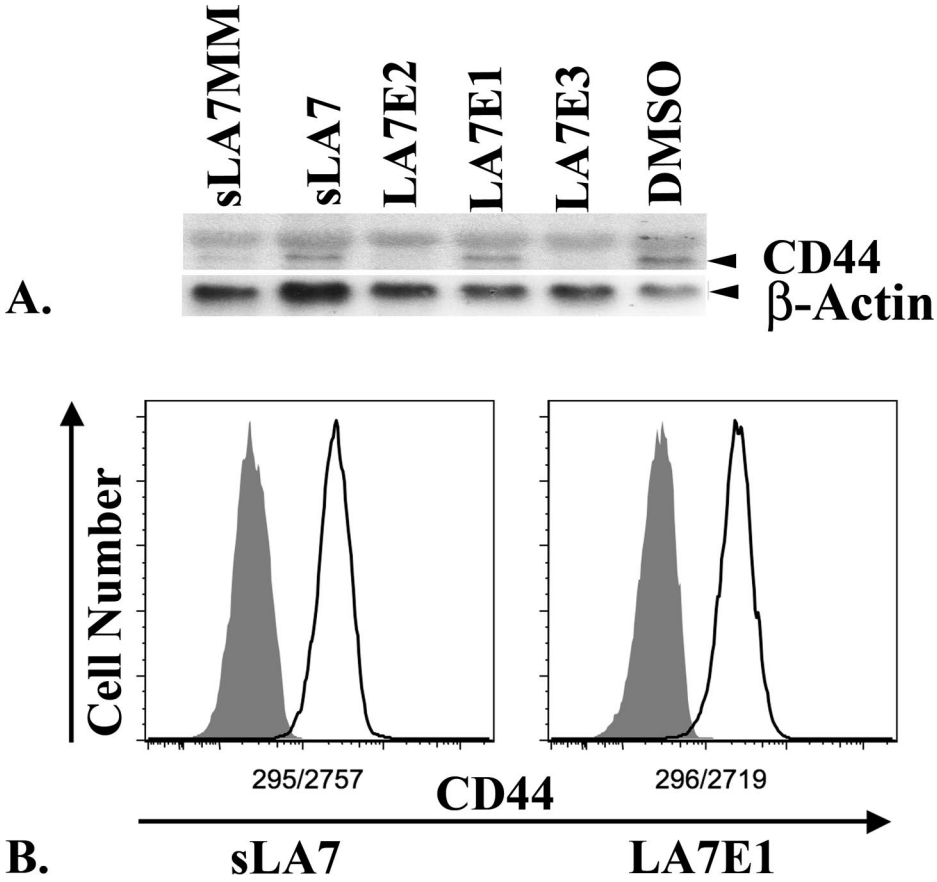

Fig. S7. CD44 expression analysis. (*A*) Northern blot analysis. CD44 mRNA is not detected in elongated LA7E2 and LA7E3 cells. (*Upper lanes*) sLA7-generated mammospheres (1), sLA7 (2), LA7E2 elongated cells derived from second-generation tumors (3), LA7E1 elongated cells derived from first-generation tumors (4), LA7E3 elongated cells derived from third-generation tumor (5), and differentiated cells derived from DMSO induction of sLA7 (6). (*Lower lane*) β-actin control. (*B*) FACS (fluorescence-activated cell sorter) analysis. CD44 expression of sLA7 and LA7E1 cells. The histograms depict CD44 and an isotype-matched control (nonfilled and gray-filled areas, respectively). The numbers indicate the mean fluorescence intensities of isotype-matched antibody/anti-CD44 antibody.

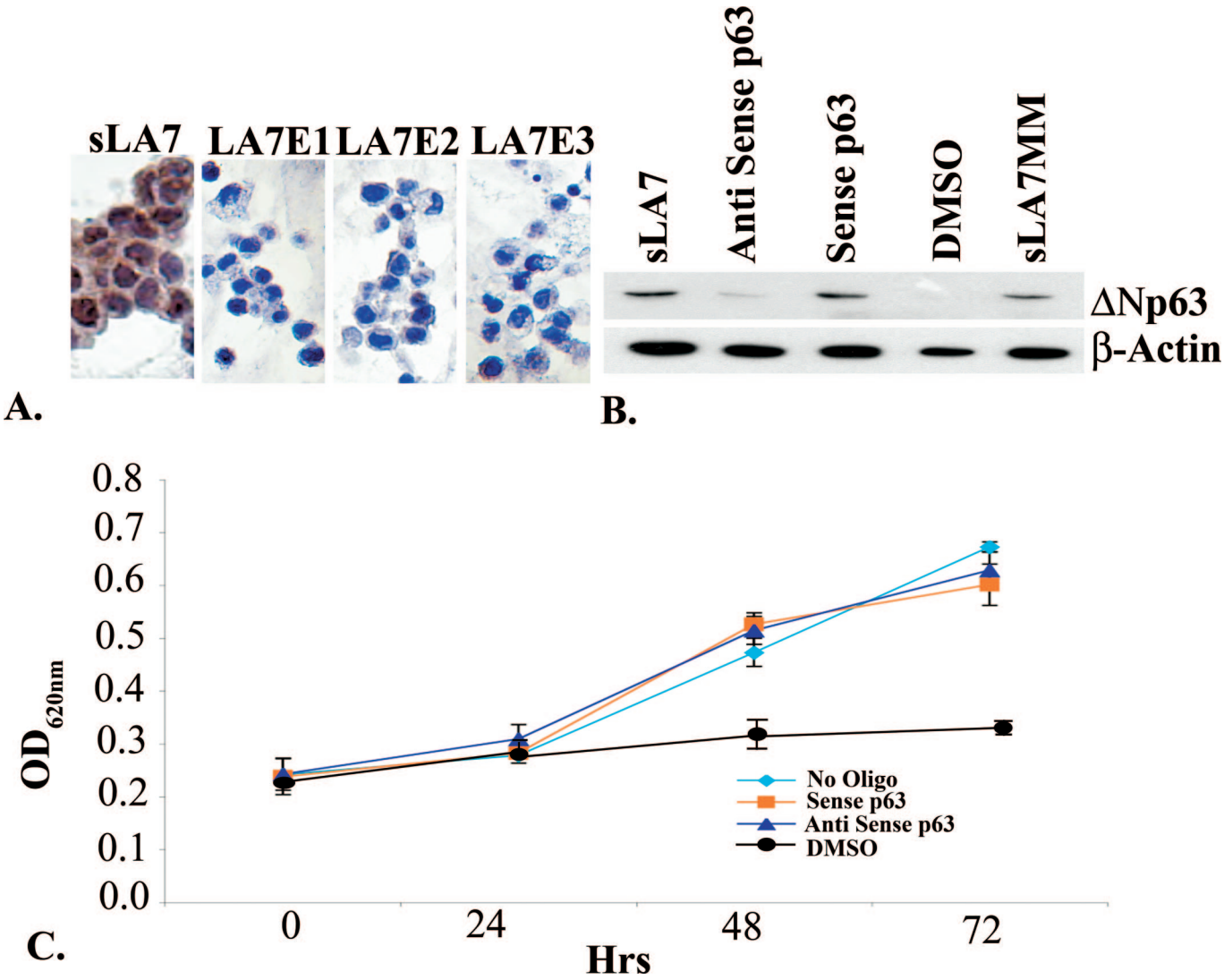

Fig. S8. p63 expression analysis. (*A*) p63 immunostaining. p63 protein (brown) is expressed in sLA7 CSCs and not expressed in LA7-elongated cells (E1, E2, and E3). Blue is Hoechst nuclear dye 33342 of nuclei. (Magnifications: sLA7, ×32; LA7E, ×20.) (*B*) p63 Western Blot analysis. p63 is expressed in sLA7, sLA7 treated with sense oligos specific for p63, and sLA7-generated mammospheres and is down-regulated in sLA7 treated with antisense oligos specific for p63 mRNA and in sLA7 induced with DMSO. (*C*) Cell proliferation measurements by MTT for adherent sLA7 cells treated with DMSO and p63 sense and p63 antisense oligos. To evaluate if sphere formation inhibition was attributable to an antiproliferative effect of the p63 oligo treatment, the cell proliferation capacity of the sLA7 cells, grown in the presence of p63 sense or p63 antisense oligos, was determined and compared with the proliferation capacity of untreated and DMSO-treated sLA7 cells. sLA7 cells were treated for 4 days as described for Fig. 5*B* and OD_{620} measurements were performed for 21 days starting at the time when the oligos or DMSO was removed from the cell cultures. No long-term changes in cell proliferation for the sense or antisense oligo-treated and untreated sLA7 cells (no oligos) were observed as compared to the DMSO-treated sLA7 cells for 21 days of culture (cells were passaged every 96 hours, shown is passage 3).

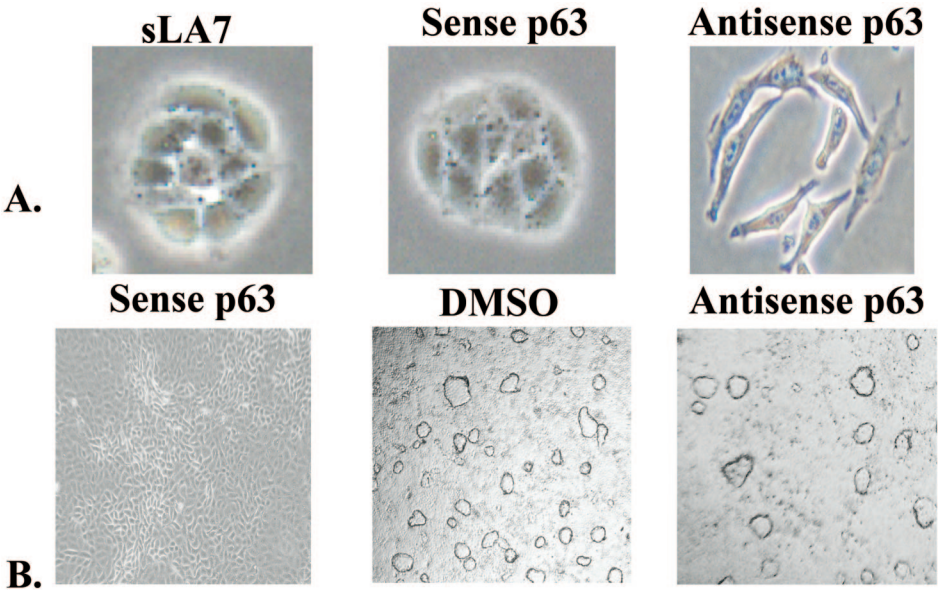

Fig. S9. Down-regulation of p63 results in the switch from self-renewing stem cells to LA7E1 progenitor cells and generation of domes. (*A*) p63 antisense oligo-treated sLA7 cells grown in non-differentiating culture conditions are associated with the complete loss of sLA7 holoclone colonies and with the generation of LA7E1 colonies. Generation of LA7E1 type cells was confirmed by using the same analyses shown in Fig. S2 *K*–*Q*. (*B*) p63 antisense oligo- or DMSO-treated sLA7 cells generate domes at 48 h. Dome formation is not induced in cells treated with p63 sense oligos.