Supplemental Data

Delineation of breast cancer cell hierarchy identifies the subset responsible for dormancy.

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Reagents and antibodies

Liquid and 10X powdered DMEM were purchased from Gibco, Grand Island, NY). Noble agar, propidium iodide, fetal bovine sera, RPMI 1640, Hoechst 33342 dye, verapamil, and mouse monoclonal IgG to β-actin were purchased from Sigma (St. Louis, MO). Biocoat Matrigel Matrix, anti-human CD44-APC, and anti-human CD24-PE were purchased from BD Biosciences (Franklin Lakes, NJ). Vybrant CFDA-SE Cell Tracer, CyQUANT Cell Proliferation Assay, Platinum SYBR Green qPCR SuperMix-UDG Kit, SuperScript III reverse transcriptase, RNase A, Platinum Taq polymerase, Dynabeads pan mouse-IgG, Connexin Antibody Sampler Pack, and Geneticin G418 were purchased from Invitrogen (Carlsbad, CA). HyGLO HRP Chemiluminescent Detection Kit was purchased from Denville Scientific (Metuchen, NJ). Restore Western Blot Stripping Buffer and NE-PER Nuclear and Cytoplasmic Extraction Kit were purchased from Thermo Scientific (Waltham, MA).

The following antibodies were purchased from Abcam (Cambridge, MA): rabbit polyclonal anti-Oct4, mouse anti-progesterone receptor (PR) mAb, rabbit polyclonal anti-estrogen receptor (ER) α, rabbit polyclonal anti-Sox2, rabbit polyclonal anti-Nanog, rabbit polyclonal anti-Musashi, rabbit polyclonal anti-ABCG2, rabbit polyclonal anti-REST and FITC-polyclonal goat anti-rabbit IgG. APC-anti-rabbit IgG and polyclonal goat anti-ribosomal protein L28 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit polyclonal IgG to acetyl-histone H3 was purchased from Upstate Cell Signaling Solutions (Lake Placid, NY). Antibodies to p15, p16, Cdk4, and cyclin D1 were purchased from Cell Signaling Technology.

Cell lines

The following cell lines were purchased from American Type Culture Collection (ATCC) and cultured as per manufacturer's instructions: Non-tumorigenic breast epithelia MCF12A; MDA-MB-231 (highly invasive, basal-like) and T47D (low-invasive, luminal). All other BCC lines were provided by Dr. Sharon Pine, Cancer Institute of New Jersey (New Brunswick, NJ).

Culture of Human Mesenchymal Stem Cells (MSCs)

Extracts from MSCs served as positive control for Oct4B (Fig. 1A). MSCs were cultured from bone marrow (BM) aspirates as described (1,2). The use of human BM aspirates followed a protocol approved by the Institutional Review Board of The University of Medicine and Dentistry of New Jersey-Newark campus. Unfractionated BM aspirates were cultured in DMEM with 10% FCS in Falcon 3003 dishes. After 3 days, red blood cells and granulocytes were removed with Ficoll Hypaque. After four cell passages, the adherent cells were asymmetric, $CD14^{-}$, $CD29^{+}$, $CD44^{+}$, $CD34^{-}$, $CD45^{-}$, $SH2^{+}$, prolyl-4-hydroxylase^{-} (1).

Flow cytometry

Intracellular flow cytometry for Oct4 was performed by the following consecutive treatments: fixed in 4% formaldehyde for 15 min at 4^0C, permeabilized in 0.1% Triton X-100 for 30 min, incubated with anti-Oct4 for 30 min at 4^0C, washed once with cold PBS and then incubated with goat anti-rabbit IgG-APC for 30 min in the dark at 4^0C. After this, cells were washed with PBS and then immediately analyzed on the FACSCalibur (BD Biosciences).

Cell surface labeling for CD44/CD24 with pEGFP1-Oct3/4 stable transfectants were performed by first washing with PBS, fixing in 4% formaldehyde as for intracellular labeling, incubating with anti-CD44-APC for 30 min followed by a second labeling with anti-CD24-PE. All incubations occurred for 30 min at 4^0C in 2% FBS/PBS. The cells were immediately analyzed by gating cells, based on green (GFP) emission, with the FACSCalibur. The data were analyzed with CellQuest software (BD Biosciences).

Side population analysis of the pEGFP1-Oct4 stable transfectants was performed using the LSR II (BD Biosciences). Stable transfectants (10^6) were washed in PBS, resuspended in phenol-free, Ca^{2+}/Mg^{2+} –free 1X Hank's Balanced Salt Solution containing 2% FBS and then incubated in titrations of Hoechst 33342 and verapamil. Optimal titrations and conditions were determined to be 5 μg/ml Hoechst 33342 (90 min incubation at 37^0C) and 400 μM verapamil (10 min pre-incubation). Cells were then washed, maintained on ice, and incubated in propidium iodide (5 μg/ml) to gate for viability. The analyses were done by gating on the top and lower 5% of GFP-expressing cells, designated $Oct4^{hi}$ and $Oct4^{-}$, respectively. The cells between the two extremes were also analyzed ($Oct4^{med}$).

Doubling time

BCCs, $5x10^3$/well, were seeded in 96-well plates. After four days cell numbers were determined by CyQUANT Cell Proliferation Assay Kit (Invitrogen). Cell numbers were calculated on a standard curve of fluorescence intensity vs. known cell densities. Calculations for doubling times were based on the following: $A = A_0*2^n$, where A = final cell number, A_0 = initial seeding density and n= number of divisions. Doubling time was taken by dividing the incubation time by the number of divisions. The fluorescence method was validated by manual cell count.

Extract preparation/Western blotting

Western blots were performed as previously described (3). Cell extracts from surgical tissues were obtained by homogenizing in the NP-40 cell lysis buffer containing protease inhibitors (Invitrogen). For intracellular proteins with cell lines, whole cell extracts were prepared with the NP-40 buffer and also nuclear/cytoplasmic extracts with NE-PER Nuclear and Cytoplasmic Extraction kit. For membrane proteins, extracts were prepared with Qproteome Plasma Membrane Protein kit (Qiagen).

BCC extracts (20 μg) were subjected to electrophoresis on 4-20% SDS-PAGE (Bio-Rad; Hercules, CA). Proteins were transferred to PVDF membranes, and membranes were incubated overnight in the respective primary antibodies. This was followed by 2 h incubation with HRP-conjugated secondary antibodies at 1:2000 final dilutions. The latter was detected with chemiluminescence. Membranes were stripped with Restore Western Blot Stripping Buffer and then re-probed for other proteins, including β-actin mAb (1:4000 dilution). All bands were normalized to β-actin.

Chemical induction of Green Fluorescence Protein (GFP) in Oct4(-) Breast Cancer Cells (BCCs)

At 40-50% confluence stably transfected BCCs were treated with G9a histone methyltransferase inhibitor, *[2-(Hexahydro-4-methyl-1H-1,4-diazepin-1-yl)-6,7-dimethoxy-N-[1-(phenylmethyl)-4-piperidinyl]4-quinazolinamine]* (BIX01294, Enzo Life Sciences, Farmingdale, NY). BIX01294 and similar chemicals can induce Oct4 in negative cells, in reprogramming (4,5). Cells were treated with 2.7 μM BIX01294. After 24 h, images were taken by fluorescence microscopy. Control cells were untreated or treated with vehicle (dimethyl sulphoxide, DMSO).

Immunohistochemistry

Oct4 staining in primary breast tissues was performed by fixing in 4% paraformaldehyde overnight followed by incubation in 20% sucrose overnight. The tissues were embedded in O.C.T. compound and then sectioned into 5 μm slices as described above. Sections (10 μM) were placed on slides and then de-paraffinized in xylene. After this, the sections were rehydrated with consecutive washes in decreasing concentrations of ethanol: 100%, 90%; 80% 70%. Slides were washed twice in PBS and then incubated in 0.25% Triton X-100 for 5 min. This was followed by blocking in 1% BSA for 1 h. Slides were incubated overnight at 4°C with anti-Oct4 (1:500 dilution). The antibody was diluted in PBS containing 0.1% Triton X-100 and 0.1% BSA. Diaminobenzidine (DAB) detection for Oct4 was performed using the DAKO Envision + System-HRP according to manufacturer's protocol.

Immunocytochemistry

BCCs were added to sterile coverslips placed within 6-well plates. The next day, after adherence, cells were washed with 1X PBS, fixed with 3.7% formaldehyde for 10 min, permeabilized with 0.1% Triton X-100 in PBS and blocked in 1% BSA in PBS for 1 h. The blocking buffer was washed with PBS and the cells were incubated with anti-Oct4 (1:500 dilution). The antibody was diluted in 0.1% BSA/0.1% Triton X-100 in PBS. After 30 min, the cells were washed in PBS and then incubated with goat anti-rabbit IgG-FITC (1:1000 dilution) for 2 h in the dark. Nuclei were stained with 300 nM DAPI, and F-actin was stained with Texas Rex-X phalloidin. Green emission was observed using the 518 nm filter.

Noble agar assay

The assay was established with two layers of noble agar in 60 mm Petri dishes. The bottom layer contained 4 mL of 0.6% agar, and the top layer contained the cells in 4 mL of 0.3% agar. The agar was prepared with a stock of 1.8% diluted in deionized water. Agar was autoclaved and then diluted to the working concentration with sterile deionized water and 2X DMEM. The bottom agar was allowed to solidify at 37^0C for 10 min. After this, the top agar was added with BCCs at concentrations between 10^1 and 10^5 at $\log_{10}$ dilutions. Plates were incubated and examined with EVOS fl fluorescence imager.

Gap junctional intercellular communication (GJIC)

GJIC was assessed by CFDA-SE dye exchange from BCCs to stroma in co-cultures, as described previously (6). Briefly, BCCs and stroma were co-cultured at equal ratios in α-MEM

with 10% FCS, in the presence or absence of 300 μM 1-octanol. CFDA-SE dye transfer was assessed on an EVOS fl fluorescence imager (AMG Micro, Bothell, WA).

Invasion assay

BD BioCoat Matrigel Matrix (0.2 ml) was added into 8 μm FluoroBlok cell culture inserts. The inserts allow for the detection of cells in the inner chambers. After solidification of matrigel, the inserts were placed in 24-well culture plates containing 0.5 mL DMEM with 10% FCS. BCCs ($2x10^4$), in sera-free media, were added to the inner wells. After 2 h at 37^0C a cotton swab was used to remove the remaining cells in the inner chambers. The migrated cells were labeled with 10 μM CDFA-SE and the incorporated CFDA was detected on a Victor 3V Multi-well plate reader (Perkin Elmer, Waltham, MA) at 485 nm/535 nm. The non-tumorigenic MCF12A served as negative control.

Cell cycle analyses

Cell cycle analyses were performed with BCCs (10^6). Cells were washed in PBS and then resuspended in 0.1% hypotonic sodium citrate solution containing 5 μg/ml propidium iodide and 200 μg/ml DNase-free RNase A. Cells were incubated for 30 min at room temperature and then immediately analyzed on FACSCalibur (BD, San Jose, CA).

Real-time PCR

RNA extraction was performed via RNeasy Mini Kit from (Qiagen, Valencia, CA). Total RNA (1 μg) were immediately reverse transcribed using dNTPs (0.2 mM), random hexamers (50 μM), and SuperScript III reverse transcriptase (200 U). Incubation conditions were 25^0C for 5 min, 50^0C for 60 min, and 70^0C for 15 min. Real-time PCR was performed with 200 ng cDNA using Platinum SYBR Green qPCR SuperMix-UDG Kit (Invitrogen) and then analyzed on the 7300 Real-Time PCR System (Applied Biosystems, Foster City, CA). The analyses were performed with an initial incubation of 50^0C for 2 min followed by 95^0C for 2 min. After this, the cycling conditions were as follows: 94^0C for 15 sec and 60^0C for 45 sec, for 40 cycles. Primer sequences are described in Table S3.

Table S1: Patients' Demographics – samples from surgical tissues

Subjects (S)	Stage	Grade	Tumor Size	Histology	ER/PR	HER2	Treatment
S1*	IIIA	Intermediate	T2	Infiltrating ductal carcinoma	Negative	Negative	None
S2	IIB	Intermediate	T3	Invasive ductal carcinoma	Unknown	Unknown	None
S3	IIIC	Intermediate	T3	Infiltrating ductal carcinoma	Positive	Positive	None
S4	IIIA	High	T2	Infiltrating ductal carcinoma	Unknown	Unknown	None
S5	IIA	High	T2	Invasive ductal carcinoma	Negative	Negative	None
S6	IIIC	High	T2	Infiltrating ductal carcinoma	Unknown	Unknown	None

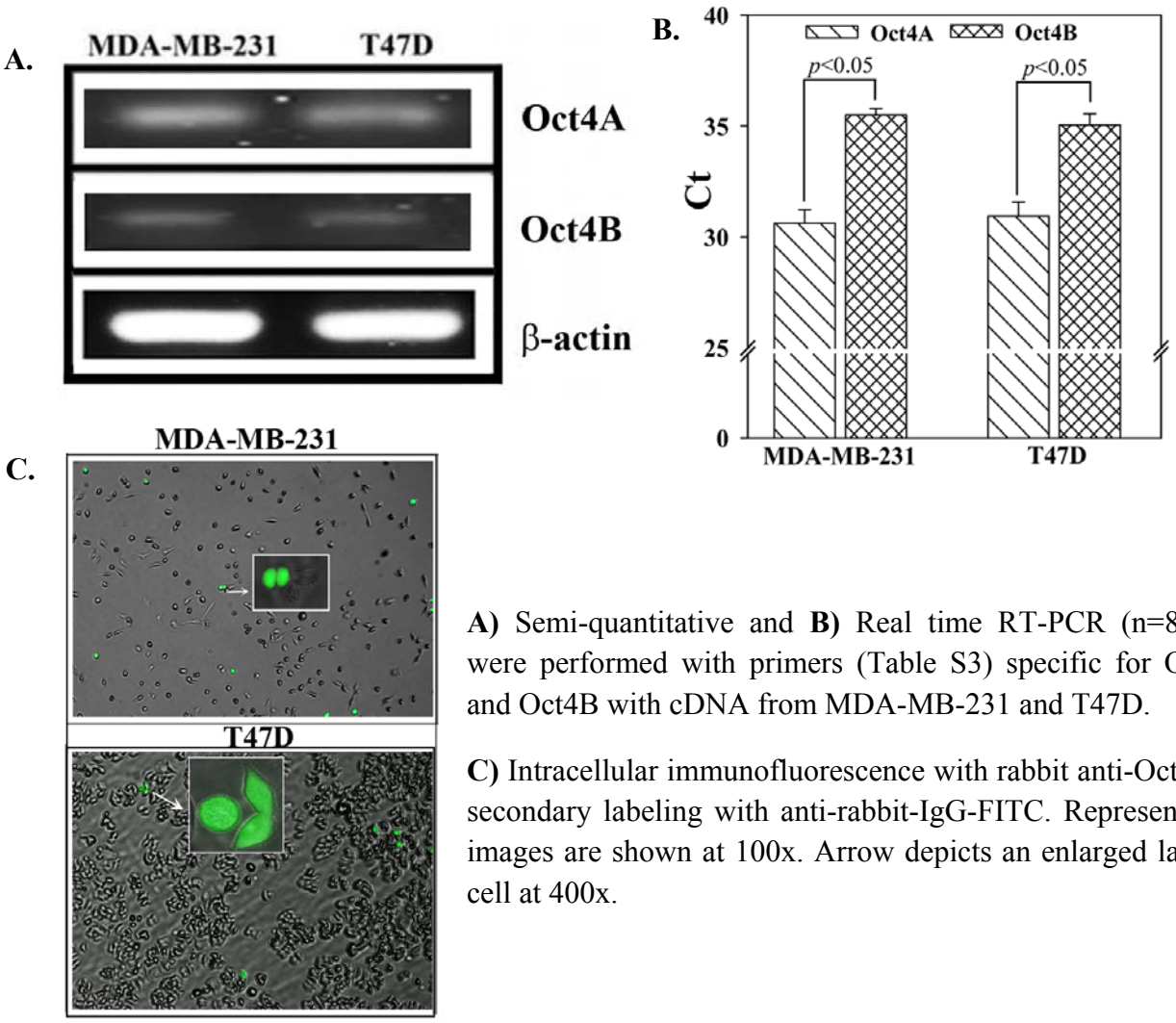
Table S2: Patient Data for Peripheral Blood Samples

Patients	Stage	ER/PR status	HER2 status	Treatment	Documented distant metastasis?	Obese	Other
P1	IIIB	Neg	Neg	None	No	No	T4 tumor 12 cm tumor; 36 neg lymph nodes
P2	III	Neg	Neg	Chemotherapy/ Radiation	No	No	
P3	IIIA	Pos	Unknown	Chemo, then surgery	No	No	Tumor and nodes present
P4	IIA	Pos	Pos	None	Yes	Yes	
P5	N/A	Pos	N/A	None	No	Yes	
P6	III	Neg	Neg	Surgery, then chemo	No	No	
P7	III	Pos	Neg		No	Yes	
P8	I	Pos	Neg				
P9	2^{nd} Rem-ission	Unknown	Unknown	Partial mastectomy/ Chemotherapy/Lymph Node dissection	Yes	No	Diagnosed, 1988; Recurrence in 1999 Currently, in remission

Table S3: Primer Sequences

Primer	Sequence (5′ → 3′)
Oct4A-Forward	TTC AGC CAA ACG ACC ATC
Oct4A-Reverse	CAG GTT GCC TCT CAC TCG
Oct4B-Forward	AAG TTA GGT GGG CAG CTT
Oct4B-Reverse	GGG TGA TCC TCT TCT GCT
β-actin-Forward	TGC CCT GAG GCA CTC TTC
β-actin-Reverse	GTG CCA GGG CAG TGA TCT

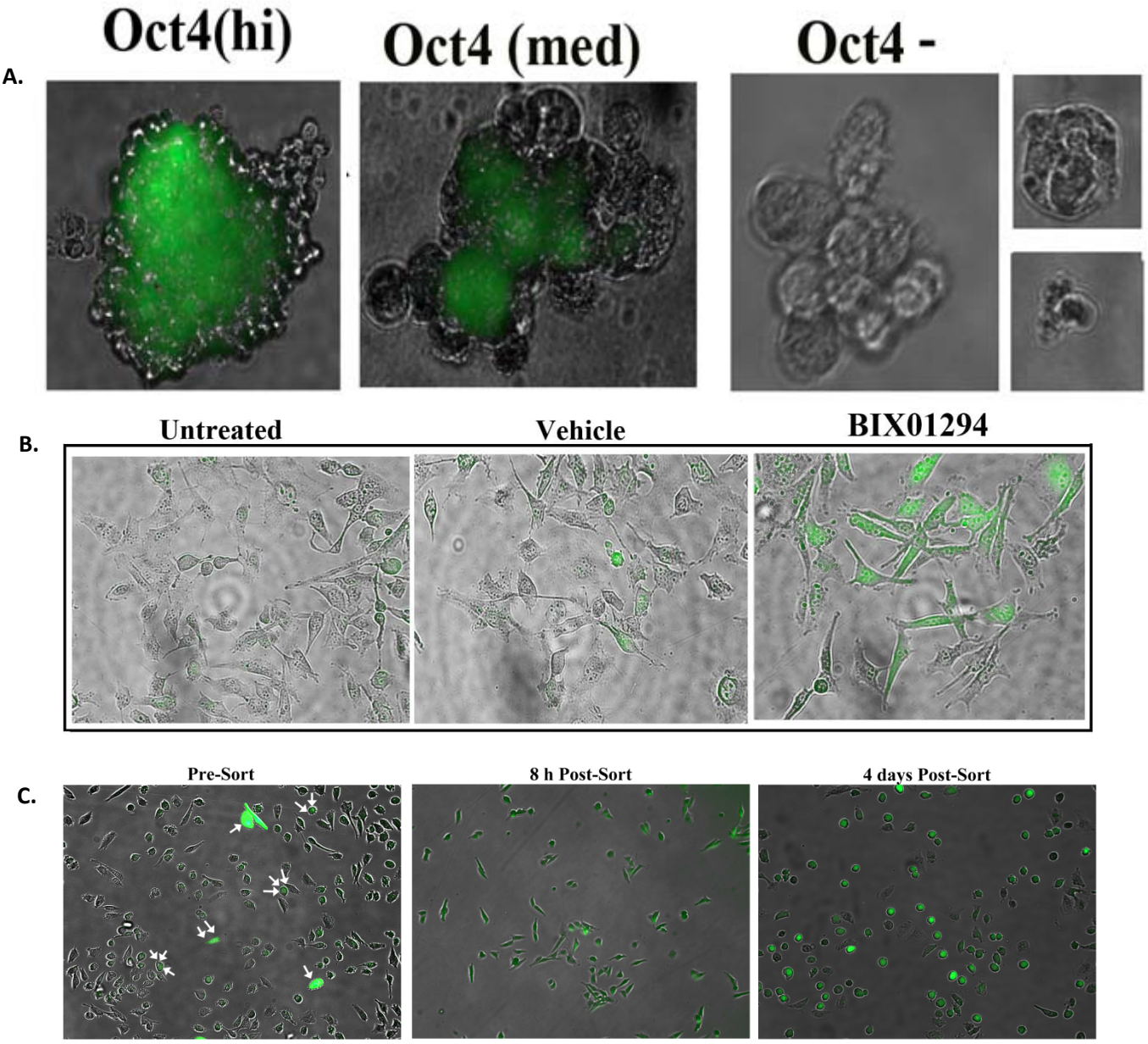
FIGURE S1



A) Semi-quantitative and **B)** Real time RT-PCR (n=8±SD) were performed with primers (Table S3) specific for Oct4A and Oct4B with cDNA from MDA-MB-231 and T47D.

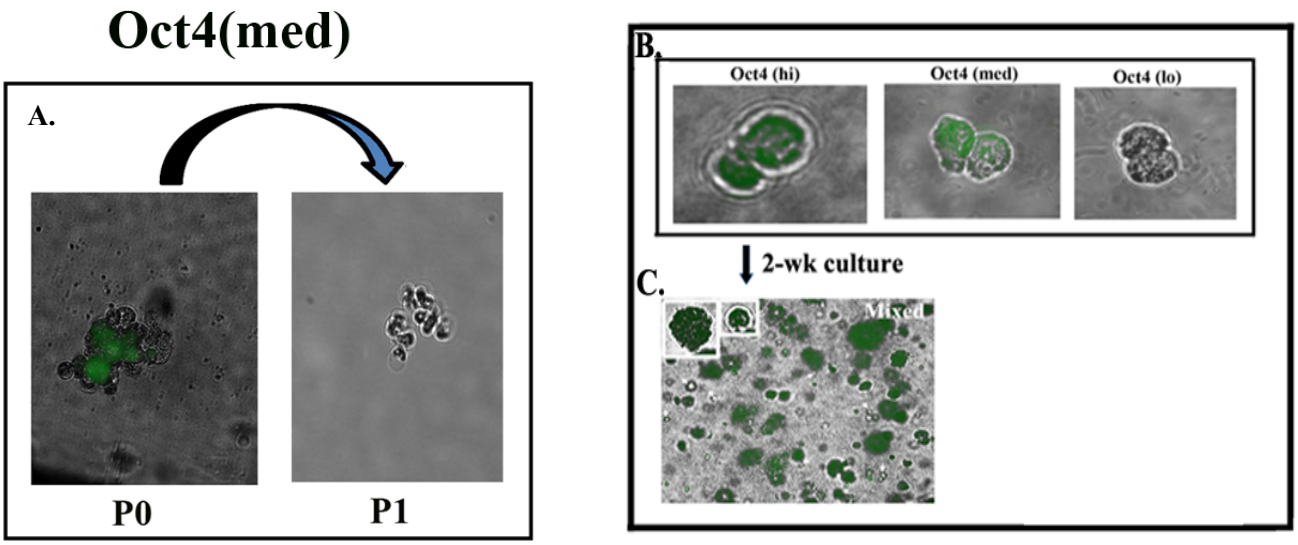
C) Intracellular immunofluorescence with rabbit anti-Oct4 and secondary labeling with anti-rabbit-IgG-FITC. Representative images are shown at 100x. Arrow depicts an enlarged labeled cell at 400x.

FIGURE S2



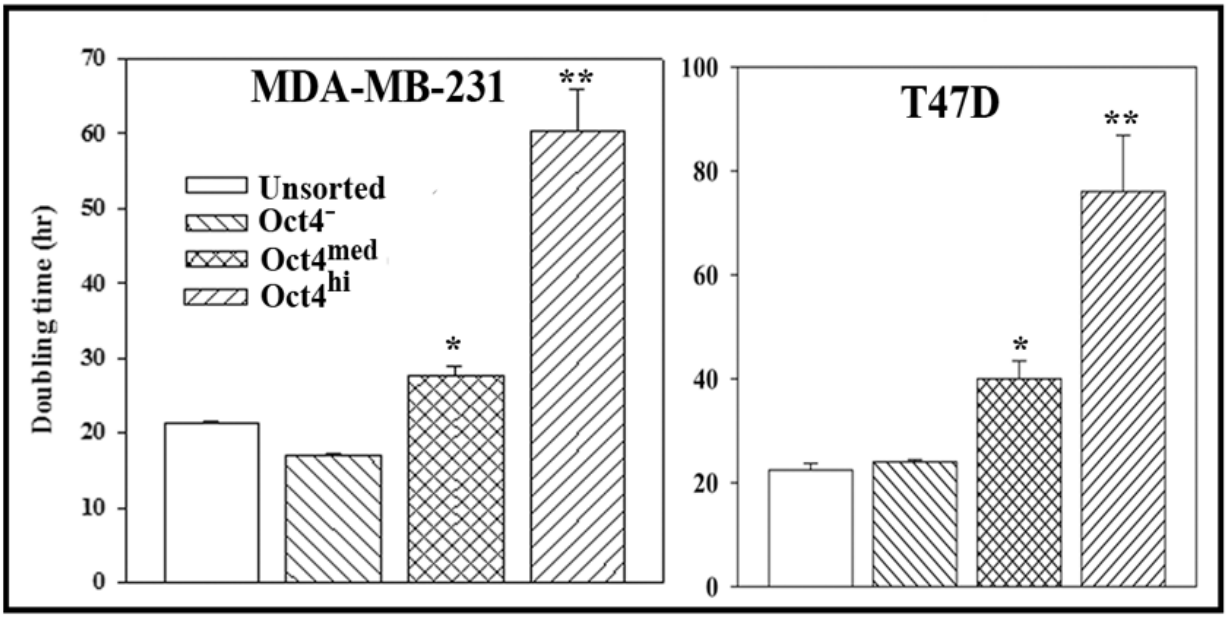
(A) Tumorspheres with BCC subsets. Shown are representative tumorspheres from the three isolated subsets. Oct4^{-} BCC failed to form tumorsphere. **(B) Treatment with Oct4 inducer.** BIX01294, a G9a histone methyltransferase inhibitor, was used to assess for Oct4 induction in stably transfected BCCs since it was previously shown to induce *Oct4* expression (5). **(C) Post-FACS purity assessment.** pEGFP1-Oct4 stable transfectants were visualized by fluorescence microscopy before and after sorting by FACS. Left: pEGFP1-Oct4 stable transfectants prior to sorting. Middle and Right panels: Oct4^{hi} BCCs were plated and images were taken at 100X magnification at 8 h and 4 days after sorting.

FIGURE S3



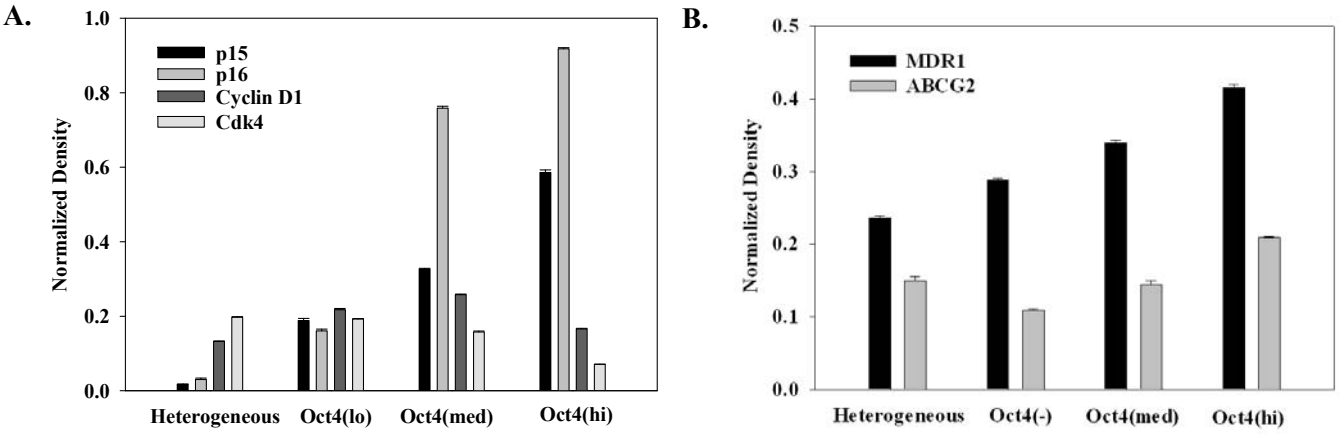
Relative maturity of BCC subsets. A) The figure shows a small tumorsphere from Oct4^{med} BCCs that failed to be serially passaged. **B)** Shown are the first doubling times for different BCC subsets. **C)** The parental and daughter from an original Oct4^{hi} cell were expanded in liquid culture. At wk 2, the cells were subcultured in noble agar. Inset shows the parental and second generation daughter cells.

FIGURE S4



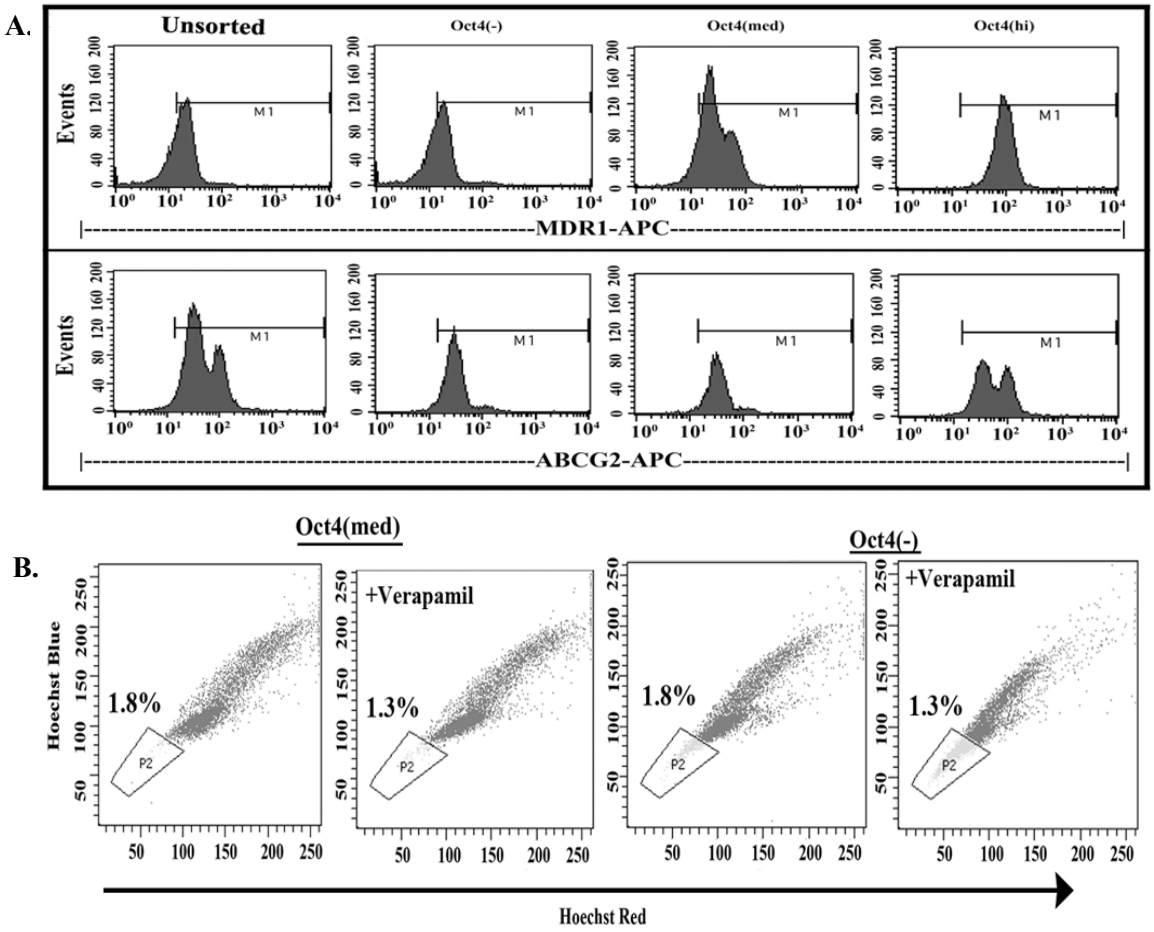
Doubling times of BCCs, based on proliferation. Freshly sorted BCC subsets were assessed for doubling times using CyQUANT Cell Proliferation Assay. The results are presented as mean±SD, n=4. ***p*<0.05 vs. other subsets; * *p*<0.05 vs. unsorted and Oct4$^-$ cells.

FIGURE S5



Densitometric analyses of western blots shown in Figs 3A (A) and 3G (B). Bands were normalized to β-actin and then presented as the mean normalized densities, ±SD, n=4.

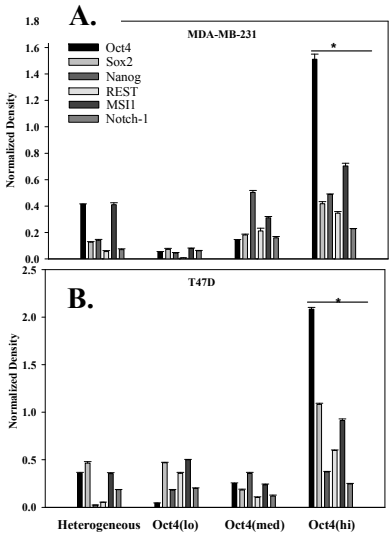
FIGURE S6

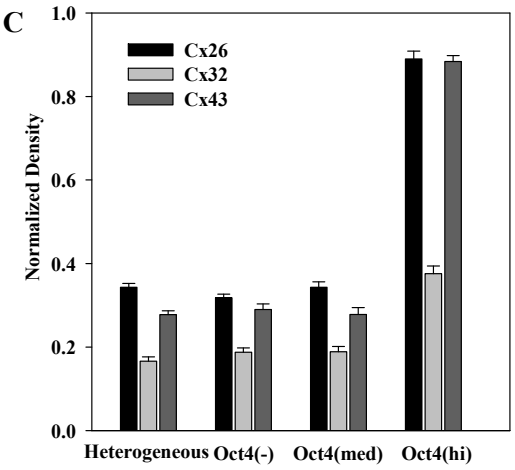


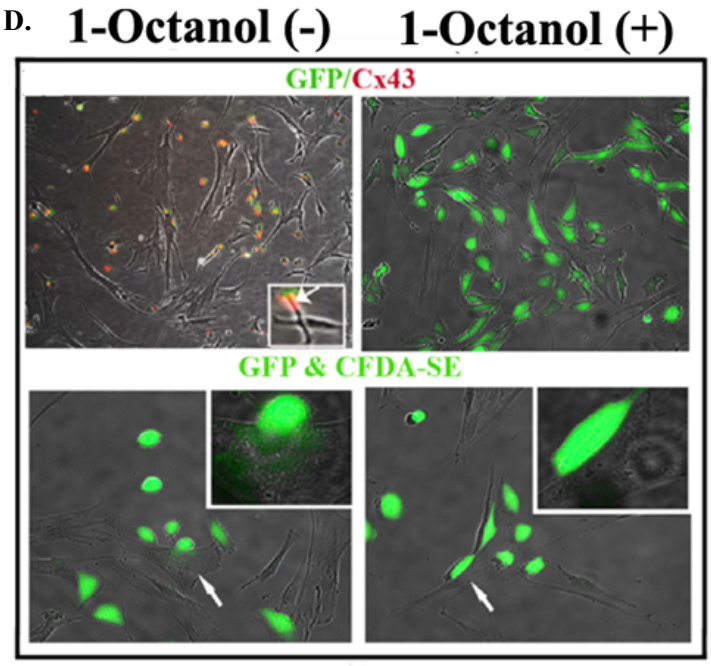
(A) Flow cytometry for ABCG2 and MDR1 in T47D. Unsorted and sorted BCCs were studied for the expression of MDR1 and ABCG2 by flow cytometry. Shown are representative of four different experiments.

(B) Dye exclusion studies. Shown are studies using $Oct4^{med}$ and $Oct4^{-}$ BCCs in dye retention studies using Hoechst 33342. The analyses were performed in the presence or absence of verapamil. The cells were analyzed on Hoechst Blue and Red filters. The figure represents five different experiments with subsets from MDA-MB-231 and T47D.

FIGURE S7





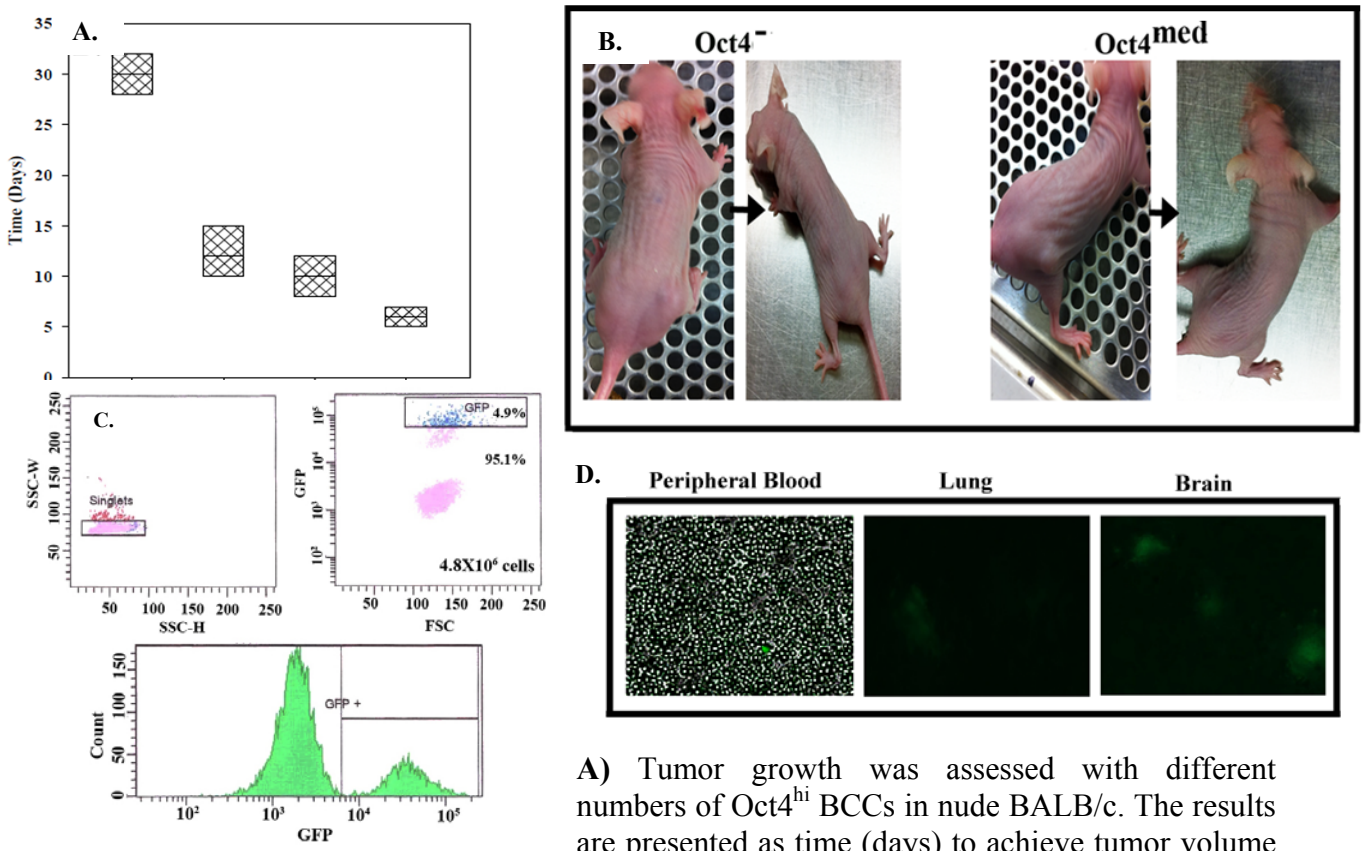


A &B) Normalized band densities of western blots for Fig. 4E. The densities of normalized bands from western blots are presented as mean±SD, n=4.

C) Normalized band densities for western blots in Fig. 5D. The normalized densities of bands are presented mean±SD, n=4.

D) Co-cultures of stroma and Oct4^{hi} BCCs. Immunofluorescence were performed for Cx43 (PE) in co-cultures with Oct4^{hi} and stroma, in the presence or absence of GJIC inhibitor 1-octanol (upper panels). The lower panels studied dye transfer with CFDA-SE-Oct4^{hi} BCCs, in the presence or absence of 1-octanol. Dye transfer was indicated by transfer of CFDA to stroma.

FIGURE S8



A) Tumor growth was assessed with different numbers of Oct4^{hi} BCCs in nude BALB/c. The results are presented as time (days) to achieve tumor volume of 0.5 cm^3, n=5; mean with upper line = 75th percentile and lower line = 25th percentile.

B) *In vivo* serial transplant of Oct4$^-$ and Oct4^{med} BCCs. 200 cells were injected in the dorsal flank of nude BALB/c. The tumors regressed after 2 weeks and were not passaged.

C) Sorting of tumors from nude BALB/c mice. Shown is a tumor, excised from a mouse injected with 200 Oct4^{hi} MDA-MB-231. The singlets were gated (top left panel) to ensure that high GFP intensity was not mistaken by doublets of cells with low GFP expressions. The top 5% of GFP singlets were sorted (top right and lower panels) for passage in naïve mice. This was done by injecting in the dorsal flank.

D) Metastasis of Oct4^{hi} BCCs to lung and brain within 24 h. BCC subsets (Oct4^{hi}, Oct4^{med}) were injected via retro-orbital route in nude female BALB/c. After 24 h, peripheral smears were examined for GFP(+) cells in crushed lung tissue and whole brain on slides. GFP(+) cells were undetectable in brain and lung of Oct4^{med} BCCs (not shown).

MOVIES

Movie S1. An Oct4$^+$ cells gives rise to daughter cells with asymmetric proliferation rates.

Bright-field and fluorescence images were done for cell divisions of a representative Oct4$^+$ cell was obtained by video time-lapse microscopy images taken every 10 min for 68 h. The parental cell is marked with a green arrow, and the progeny of the first and second divisions are marked with orange and red arrows, respectively. The Oct4$^+$ cell undergoes its first cell division 7 h after the start of time-lapse imaging, and the cell cycle times of the daughter cells were markedly different. One daughter divides at 38 h, whereas the other daughter does not divide until 64 h. The cell division at 38 h resulted in three cells, and one of the cells underwent cell death (marked by a yellow X).

Movie S2. An Oct4$^-$ cell gives rise to daughter cells with symmetric proliferation rates.

Bright-field of cell divisions of a representative Oct4$^-$ cell was obtained by video time-lapse microscopy images taken every 10 mins for 68 h. The parental cell is marked with a green arrow, and the progeny of first, second and third cell divisions are marked with orange, red and blue arrows, respectively. The Oct4$^-$ cell undergoes the first cell division at 2 h after the start of time-lapse imaging, and the second division and the cell cycle times of the progeny in the lineage tree are similar. The second and third divisions of all the progeny occur by 30 hand 59 h, respectively.

Decalcification and processing of murine femurs

Mice were injected intravenously with 10^3 BCCs, stably transfected with pEGFP1-Oct3/4. After 24 h, mice were injected, intraperitoneally with carboplatin (50 mg/kg), followed by a second dose after 3 days. At one week after the final injection of carboplatin, mice were euthanized, and the femurs were removed. Femurs were rinsed and the cells flushed using a 27 gauge needle attached to a syringe with PBS to remove the cells within the central region of the cavity. After this, the femurs were fixed overnight in 4% formaldehyde at 4°C. After this, the femurs were transferred to decalcification solution (Cal-Ex Decalcifier, Fisher Scientific, Pittsburgh, PA) overnight at 4°C. After this, femurs were rinsed in running dH2O for 4 h and then embedded in Optimal Cutting Temperature (O.C.T.) compound (Tissue-Tek, Redding, CA). After this, tissues were section in 10 μm with a cryostat-microtome HM550 (Walldorf, Germany). Slides were examined with an EVOS fl fluorescence imager.

References

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