Stem Cell Reports, Volume *2*

Supplemental Information

Growth Hormone Is Secreted by Normal Breast Epithelium upon Progesterone Stimulation and Increases Proliferation of Stem/Progenitor Cells

Sara Lombardi, Gabriella Honeth, Christophe Ginestier, Ireneusz Shinomiya, Rebecca Marlow, Bharath Buchupalli, Patrycja Gazinska, John Brown, Steven Catchpole, Suling Liu, Ariel Barkan, Max Wicha, Anand Purushotham, Joy Burchell, Sarah Pinder, and Gabriela Dontu

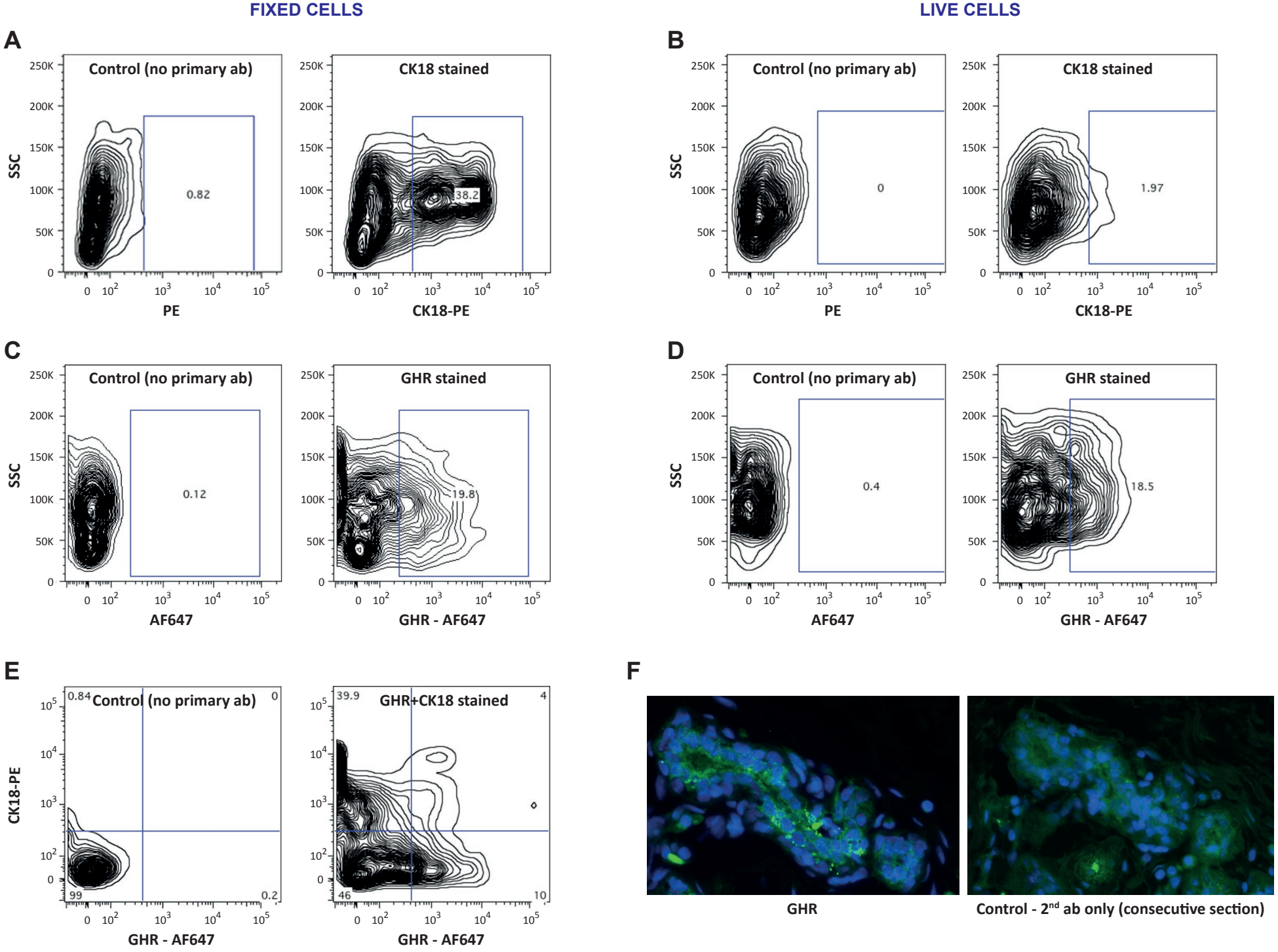
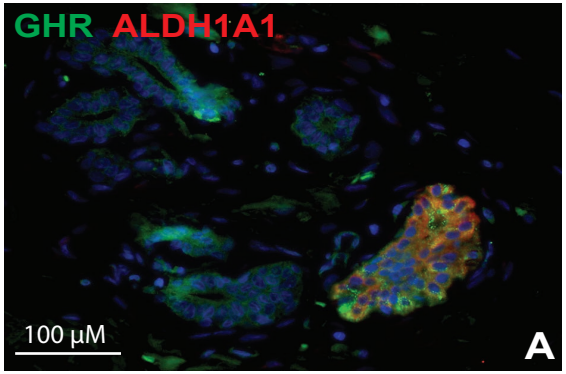
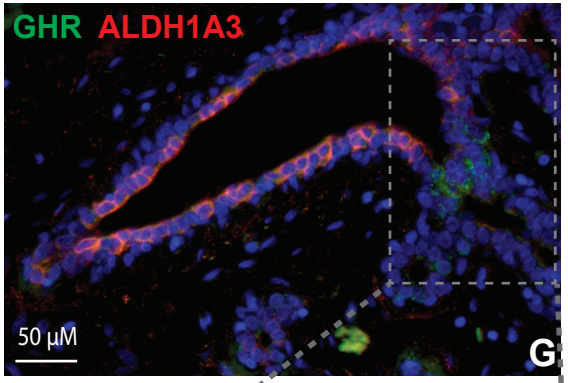
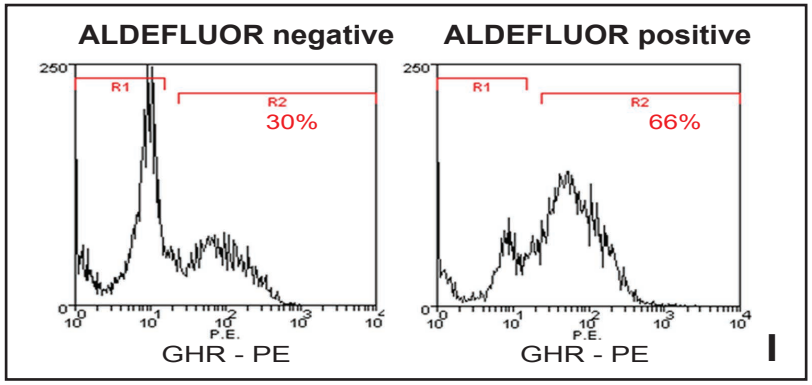
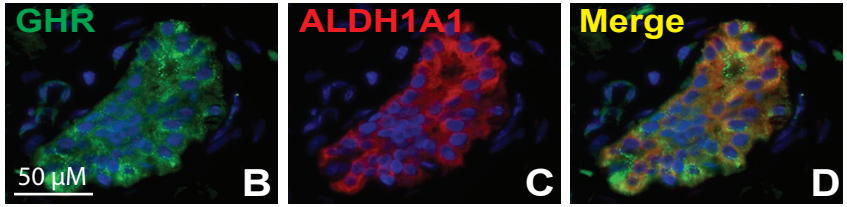

FIXED CELLS
LIVE CELLS
A
Control (no primary ab)
CK18 stained
0.82
38.2
SSC
PE
CK18-PE
B
Control (no primary ab)
CK18 stained
0
1.97
SSC
PE
CK18-PE
C
Control (no primary ab)
GHR stained
0.12
19.8
SSC
AF647
GHR - AF647
D
Control (no primary ab)
GHR stained
0.4
18.5
SSC
AF647
GHR - AF647
E
Control (no primary ab)
GHR+CK18 stained
0.84
0
99
0.2
39.9
4
46
10
CK18-PE
GHR - AF647
GHR - AF647
F
GHR
Control - 2nd ab only (consecutive section)

Figure S1

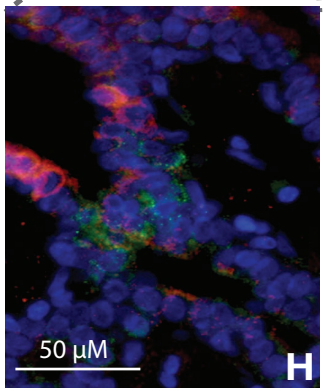


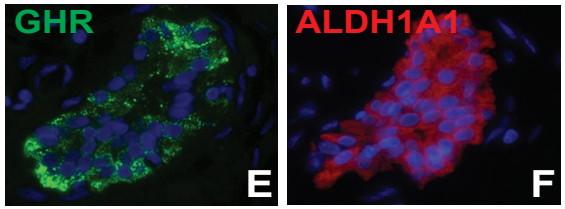




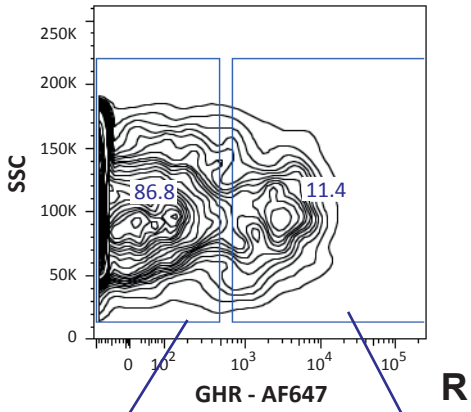


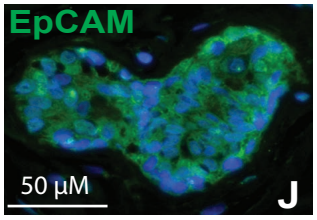
Double immunostaining

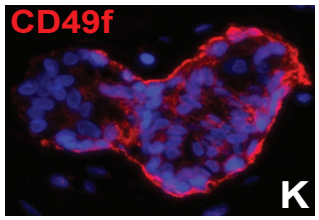


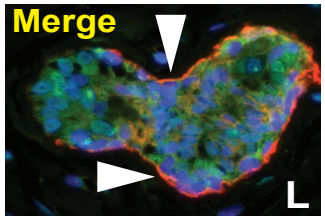


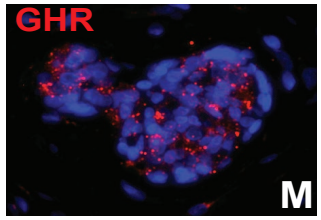
Single immunostaining on consecutive sections





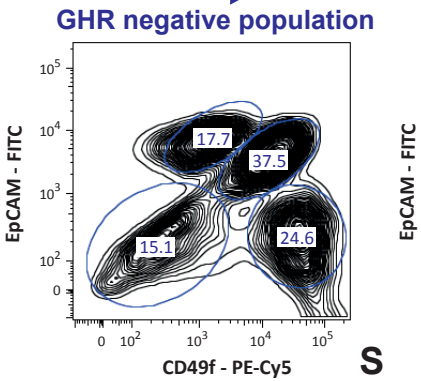


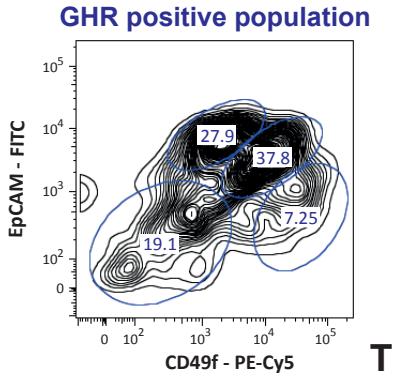


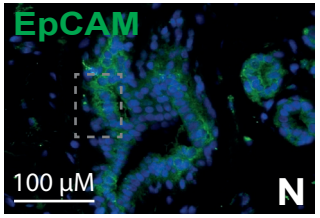


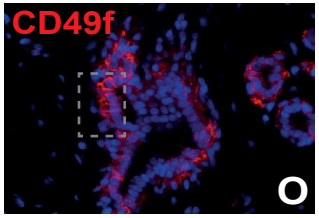
Double immunostaining

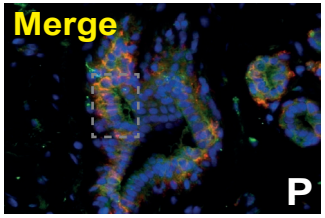
Single immunostaining on consecutive section

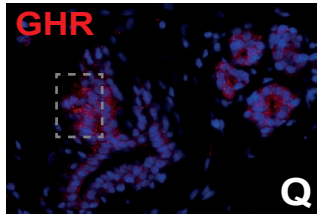








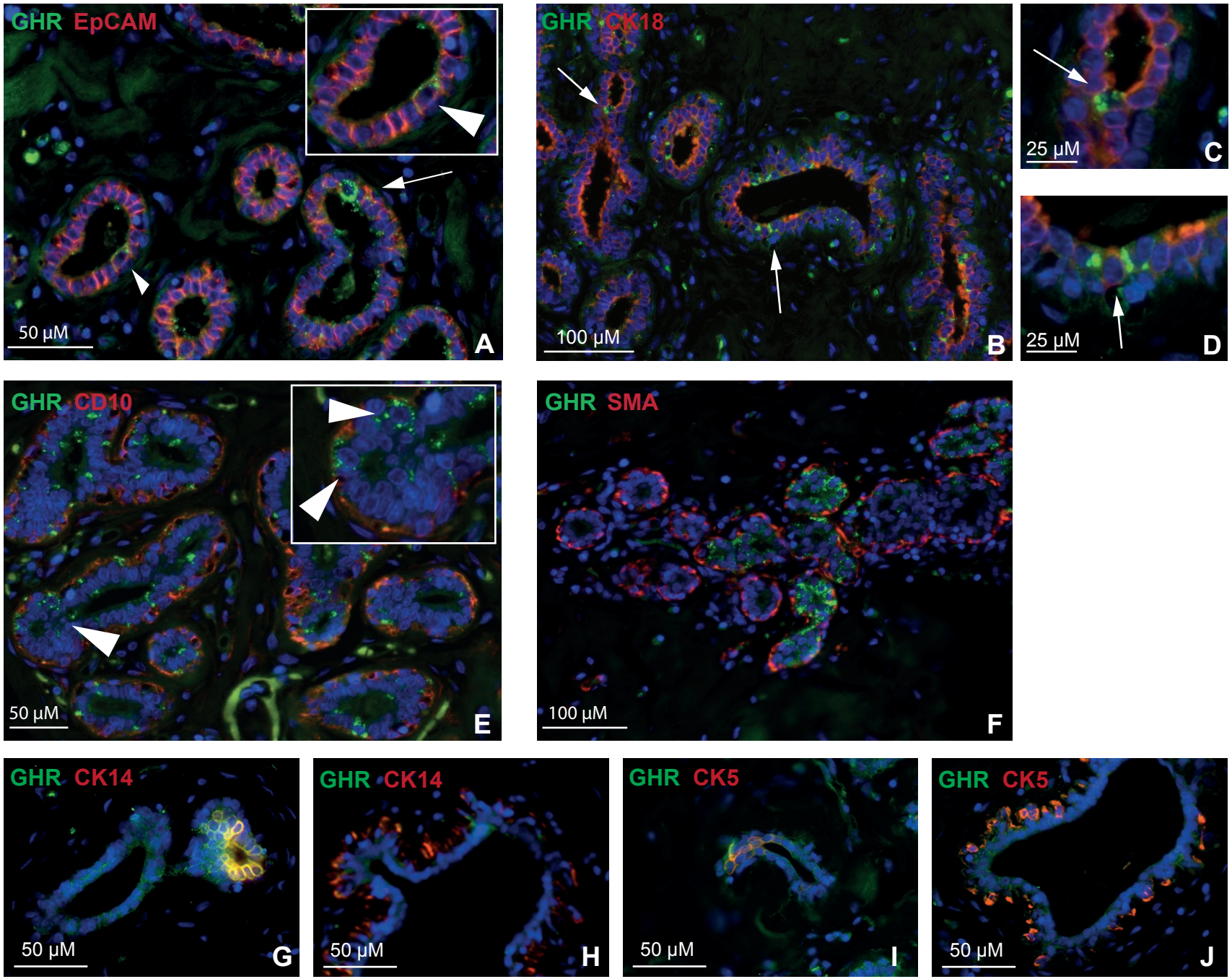




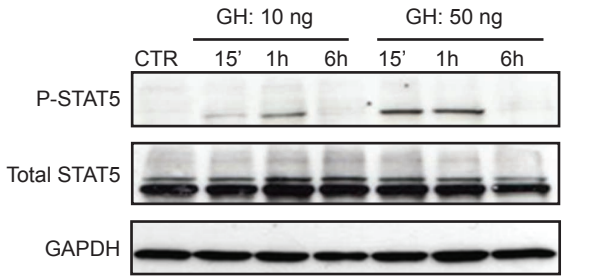
Double immunostaining

Single immunostaining on consecutive section

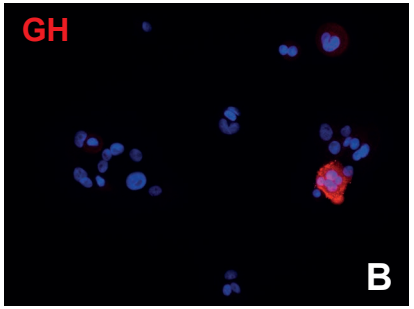
Figure S2

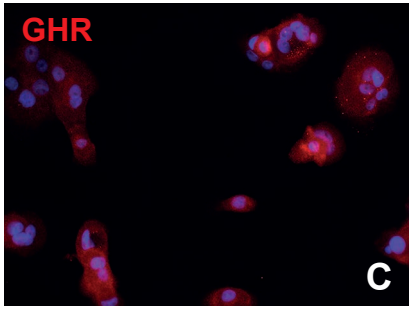

GHR EpCAM
50 μM
A
GHR CK18
100 μM
B
25 μM
C
25 μM
D
GHR CD10
50 μM
E
GHR SMA
100 μM
F
GHR CK14
50 μM
G
GHR CK14
50 μM
H
GHR CK5
50 μM
I
GHR CK5
50 μM
J

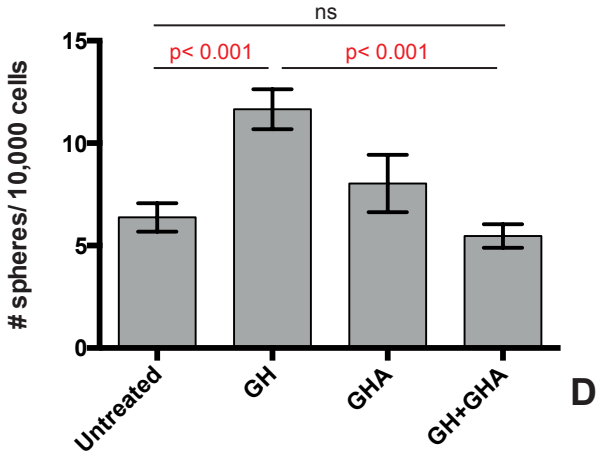
FigureS3



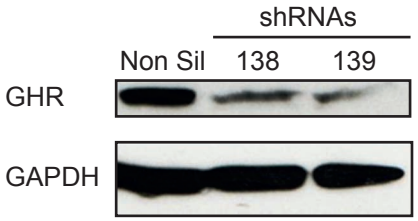
A



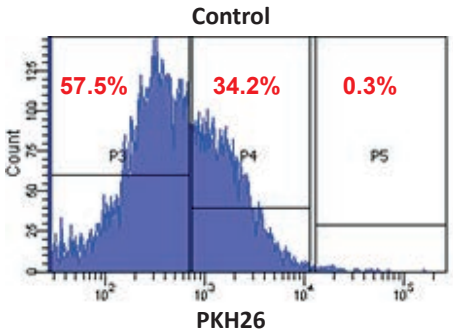


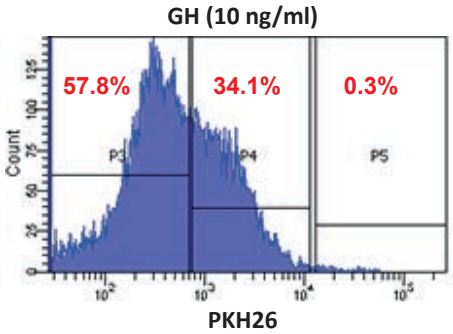


D

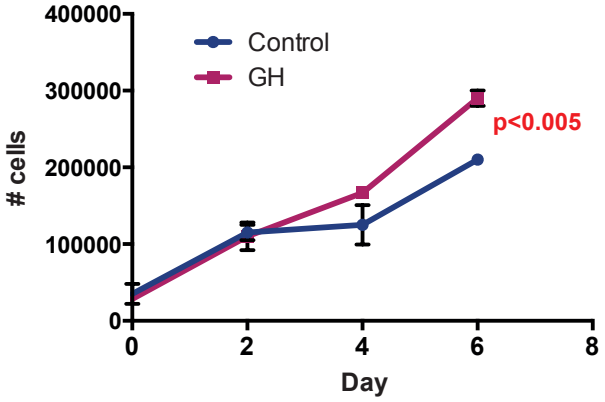


E

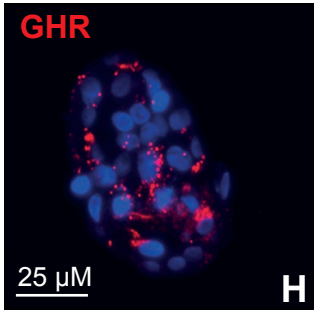


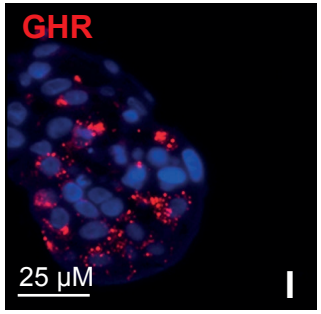


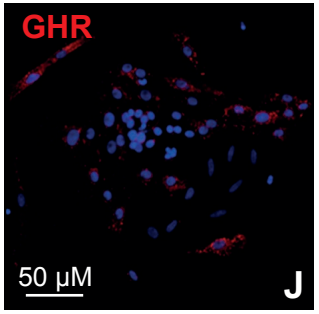
F



G







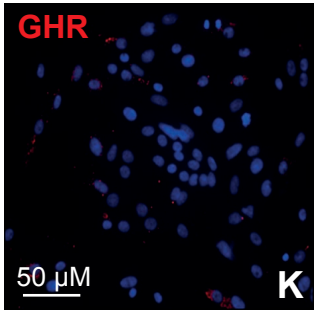
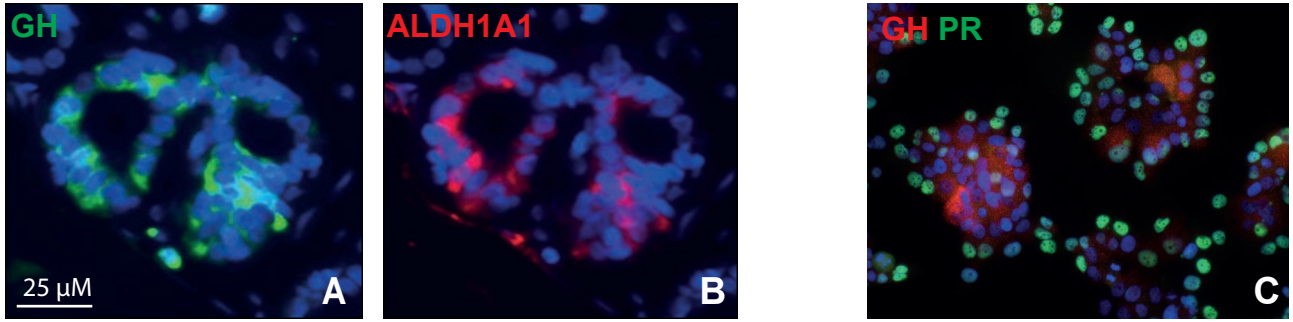
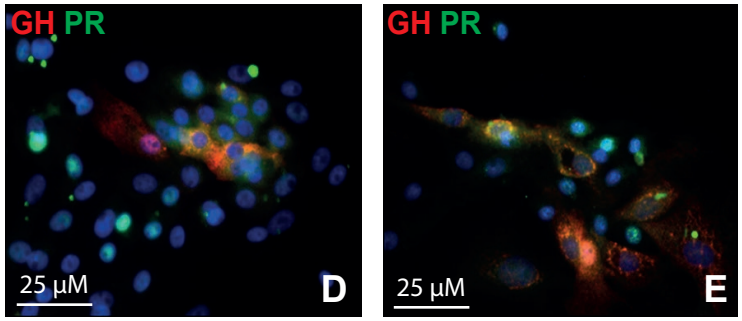


Figure S4

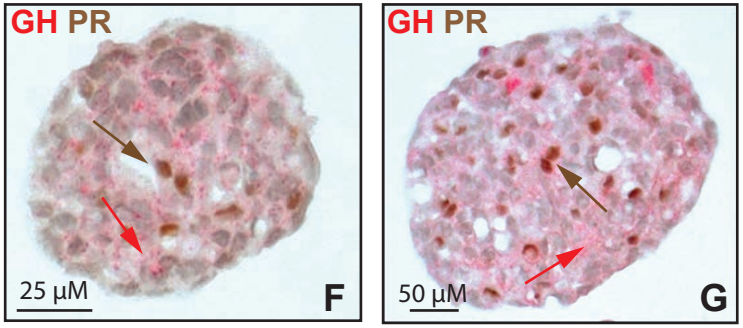
In situ IF of normal breast tissue sections



IF of human normal mammary epithelial cells in culture



IHC of mammosphere sections



FigureS5

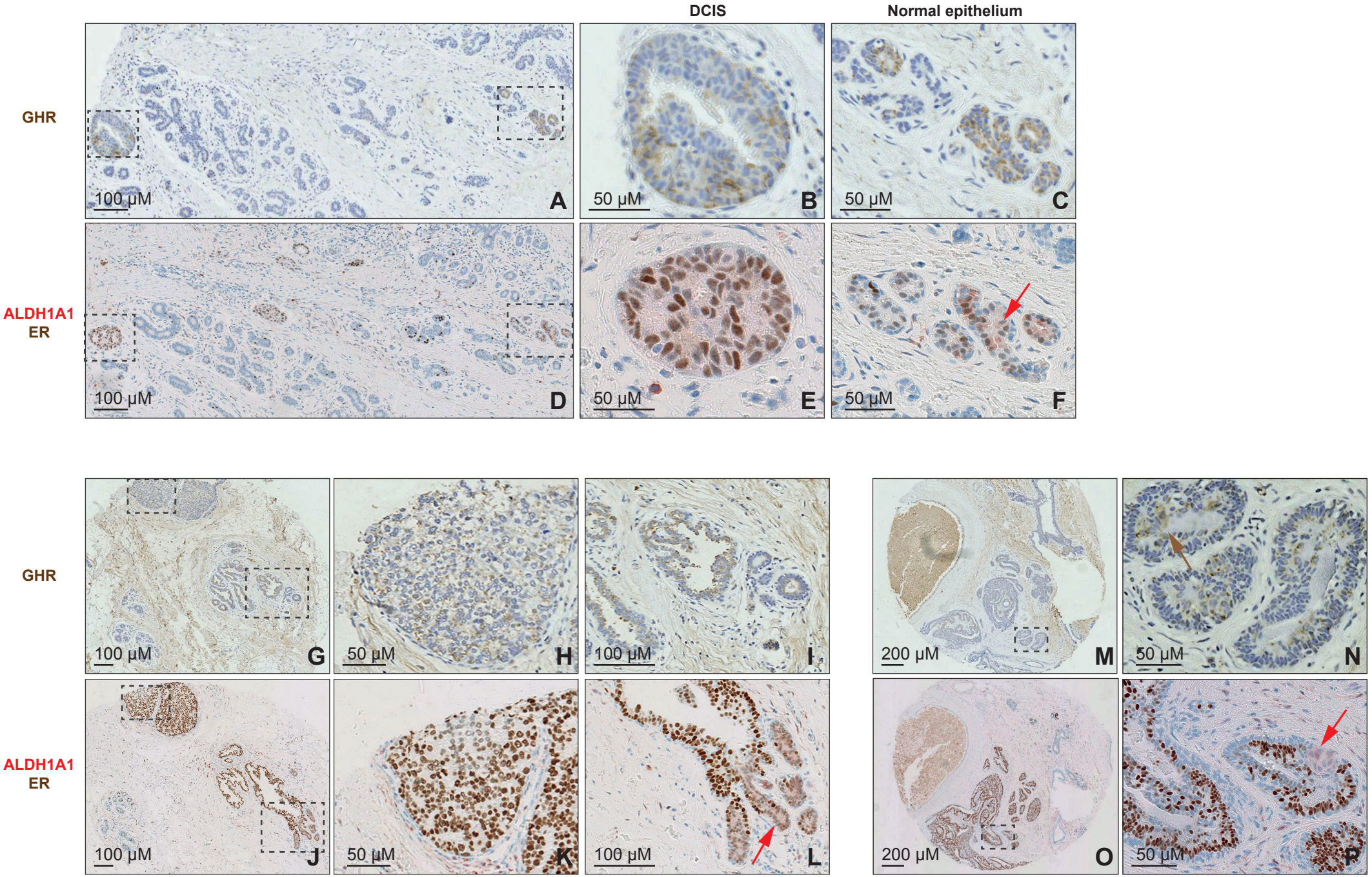

DCIS
Normal epithelium
GHR
ALDH1A1
ER
100 μM
A
50 μM
B
50 μM
C
100 μM
D
50 μM
E
50 μM
F
GHR
ALDH1A1
ER
100 μM
G
50 μM
H
100 μM
I
200 μM
M
50 μM
N
100 μM
J
50 μM
K
100 μM
L
200 μM
O
50 μM
P

Figure S6

SUPPLEMENTAL FIGURE LEGENDS

Figure S1. Control staining for flow cytometry analysis. Immunostaining for CK18 and GHR using live cells and fixed cells. Fixation did not change the representation of GHR positive cells in single analysis and in double staining with CK18. As expected, CK18 did not stain live cells unless they were permeabilized by fixation. Samples for which primary antibody was eliminated from staining did not show a positive cell population, indicating that the secondary antibody did not generate any background. These samples were used to establish the gates for analysis. Similar controls were used in all flow cytometry analyses and FACS, in addition to cells stained for viability alone and cells stained with one primary antibody only (single immunostaining). Example of immunostaining with GHR and secondary antibody alone in consecutive sections, to assess background generated by non-specific IF staining in situ. Similar controls were performed for all the antibodies used (data not shown).

Figure S2. GHR is expressed in a subset of mammary stem/progenitor cells. **A-D.** Double immunostaining for GHR and ALDH1A1 on normal breast sections. The majority of GHR+ cells are either ALDH1A1+ or situated in proximity of ALDH1A1+ cells. **E-F.** Single immunostaining with GHR and ALDH1A1 on sections consecutive to those shown in A-C is shown. **G-H.** Double immunostaining on normal breast section showing GHR+ cells adjacent to ALDH1A3+ cells. **I.** ALDE+ and ALDE- human mammary epithelial cells freshly isolated from reduction mammoplasties were separated by FACS then fixed, stained for GHR (PE) and analyzed by flow cytometry. Representative example showing 66% GHR+ cells in the ALDE+ cell population is shown. **J-Q.** Immunostaining of consecutive sections of normal breast epithelium for GHR and double immunostaining for EpCAM and CD49f markers shows GHR+ cells in proximity of CD49f+EpCAM- cells (L, arrowheads) and CD49f+EpCAM+ cells (N-Q). **R-T.** Flow cytometry for CD49f/EpCAM in GHR+ and GHR- populations shows a small overlap of the CD49f+EpCAM- stem cell phenotype with the GHR+ population (0.7% of the total cell population) and an overlap with the luminal progenitor cell population CD49f+EpCAM+ with the GHR+ cell population (5% of the total cell population).

Figure S3. GHR expression is partially overlapping with lineage markers. A-D. Double staining for GHR and luminal markers ESA or CK18, showing rare overlapping cells (arrowhead). **E-F.** Double staining for GHR and myoepithelial markers CD10 and SMA shows no overlap between either of these markers and GHR. **G-J.** Double staining for GHR and CK14 or CK5 shows co-localization of GHR with CK14 and CK5 in intralobular cells, in the luminal layer (G, I), but no co-localization with these cyokeratins, in bigger ducts, where CK14 and CK 5 are present in the basal layer (H, J).

Figure S4. GH increases proliferation of human mammary epithelial cells. A. WB analysis of Stat5 phosphorylation status upon GH treatment. MCF10A cells were treated with GH (10 and 50 ng/ml) and collected at 15 min, 1h and 6 h. A dose and time response effect on Stat5 phosphorylation was detected. The phosphorylated Stat5 was normalized with respect to the total Stat5 protein level. **B.** Immunofluorescent staining for GH in MCF10A cells in culture. **C.** Immunofluorescent staining for GHR in MCF10A cells in culture. **D**. Mammosphere formation in MCF10A cell culture was increased upon treatment with GH, but did not change after treatment with GHA or GH+GHA (three independent experiments, each performed in triplicate, n=3). **E.** WB analysis of GHR protein level shows the efficiency of GHR kd obtained with two hairpins sh(138) and sh(139), compared to non-silencing control (ns). Levels were normalized to GAPDH loading control. **F**. GH treatment did not change the distribution of PKH26 high, PKH26 low and PKH26 negative cells in mammospheres from fresh mammoplasty samples. After tissue dissociation, cells were stained with PKH26 and allowed to generate mammospheres in the presence of GH (10 ng/ml) (right panel), or vehicle alone (control, left panel). After 10 days in culture, mammospheres were dissociated and analyzed by flow cytometry. The ratio PKH26 high/PKH26 low/PKH26 negative cells was not changed by GH treatment, although the number of spheres was increased as shown in Figure 3 (representative example from three independent experiments on two mammoplasty samples). **G.** GH treatment (10 ng/ml) of primary human mammary epithelial cells from a different mammoplasty sample than shown in Figure 3G (culture on collagen substratum for 6 days) resulted in increased cell proliferation (two independent experiments, each performed in triplicate, n=3). **H-K.** Immunostaining for GHR on mammopsheres (H, I) or on primary human mammary epithelial cells grown on collagen substratum (J, K) shows higher representation of GHR+ cells in mammospheres compared to cells cultured in adherent conditions, at high density.

Figure S5. GH secretion by human normal mammary epithelial cells. A, B. In situ double immunostaining for GH and ALDH1A1 on normal breast sections. Single stainings corresponding to Figure 4C merged image. **D**. Double-staining for GH and PR on T47D cells. **E, F** Double staining for PR and GH of primary human mammary epithelial cells treated with P4 (10nM (E) or 40 nM **(**F)) showing rare PR+ cells secreting GH. **G, H.** IHC for GH (red) and PR (DAB, brown) on mammosphere sections.

Figure S6. GHR, ALDH1A1 and ER immunostaining in DCIS. A-P. Immunostaining for GHR and ALDH1A1/ER on consecutive sections of DCIS samples on TMAs. Co-localization of ALDH1A1 (cytoplasmic red) and GHR (cytoplasmic brown) was observed in DCIS areas (B, E) and in normal adjacent tissue (C, F). GHR frequently co-localized with ER (nuclear brown) in areas of DCIS (H, K). Expanded GHR+ and ER+ cells adjacent to ALDH1A1 positive cells in areas of normal epithelium adjacent to DCIS lesion (red arrows) (I, L). **M-P.** DCIS area in which GHR, ER and ALDH1A1 are expressed. GHR appears to be present in cells with higher grade nuclear morphology ((brown arrow) (N). ALDH1A1 is present in ER- negative cells with similar higher grade nuclear morphology (red arrow) in consecutive sections through the same core (P).

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Tissue dissociation

To generate a single cell suspension for the *in vivo* studies, a 6h collagenase (Worthington) digestion was used. All samples were depleted of fibroblasts by differential centrifugation and assessed for stroma contamination by plating an aliquot on collagen substratum at three different densities and examining cell and colony morphology in five day cultures (Ginestier et al., 2007). Samples with stromal contamination higher than 2% were either discarded or re-digested and separated by another round of differential centrifugation, then tested again for stromal contamination.

Mammosphere paraffin embedding

Mammospheres were cultivated as previously described (Dontu et al, 2003, Cicalese et al., 2009;

Pece et al., 2010). After a week, mammospheres were gently centrifuged at 200 rpm for 2 min at RT. The pellet was then re-suspended in formalin and allowed to fix for 1h at RT. After fixation, mammospheres were centrifuged and part of the supernatant was removed leaving approximately 500 µl of fixative in the tube. 500 µl of melted 4% agarose (Gibco electrophoresis grade) in distilled water was then added to the formalin-fixed pellet (to a final agarose solution of 2%). The volume was aspirated in a 1 ml syringe and allowed to cool for 30 min at RT. The top of the syringe was then carefully cut off and the gel was expelled using the syringe plunger. The gel was wrapped in lens tissue and paraffin-wax processed following routine tissue processing procedures.

Flow cytometry.

In all experiments using flow cytometry, cells were pre-gated on forward scatter (single cells) and viability (PI or eFluor780-negative cells, Sigma and eBiosciences) Immunostaining with secondary antibodies alone were used as negative controls in experiments using immunostaining. Staining on fixed cells were performed after sorting for viability, followed by fixation in 1% PFA (20' on ice) and permeabilization with 0.1%saponin+1%BSA (30' RT). Blocking and staining with primary and secondary antibodies were performed in Rat IgG 1:200+0.1% saponin+1% BSA, 30 min for primary antibodies and 20 min for secondary antibodies, on ice.

ALDEFLUOR assay

Cells obtained from freshly dissociated normal breast epithelium were suspended in ALDEFLUOR assay buffer containing ALDH substrate (BAAA, 1 µmol/l per $1x10^6$ cells) and incubated for 40 minutes at 37°C. In each experiment a sample of cells was stained under identical conditions, in the presence of specific ALDH inhibitor diethylaminobenzaldehyde (DEAB), 50mmol/L, as negative control. All reagents were from StemCell Technologies. Cells were gated based on forward scatter plot and emission in the FITC channel, set to less than 0.1% in the negative control.

PKH26 staining

Single cells obtained from dissociation of mammoplasty samples, separated from stromal cells as previously described, were labeled with PKH26 (Sigma, 10^{-7} M for 5 min) according to

manufacturer's protocol and plated in suspension at 5,000 cells/ml to generate primary spheres, in presence of 10 ng/ml GH or vehicle control. After 7-10 days, mammospheres were harvested, dissociated enzymatically and the single cell suspension was subjected to flow cytometry analysis by BD LSR Fortessa to assess representation of PKH26high, medium and low cells (Cicalese et al., 2009, Pece et al., 2010).

Culture conditions

MCF10A cells were grown in DMEM/F12 phenol-red free supplied with 5% Horse Serum, EGF (20 ng/ml), hydrocortisone (0.5 mg/ml), cholera toxin (100 ng/ml) and insulin (10 µg/ml). 293T cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum. T47D cells were grown in RPMI with 10% insulin (5 µg/ml). All reagents were from Gibco.

GHR knockdown

Lentiviral vectors (pGIPZ) expressing two selected shRNAs against Growth Hormone Receptor (GHR) (Applied Biosystems) were used to down regulate the expression of the receptor. The vectors were first transfected into 293T packaging cell line by standard calcium phosphate precipitate method. After 36h, the supernatant containing lentivirus was used to infect primary HMECs in suspension, in serum-free medium. Two days of infection were performed (two cycles each day) at the end of which cells were checked for GFP expression by microscopically analysis. Cells were then plated at clonogenic density (5,000 cells/ml) to generate mammospheres in presence of GH (10 ng/ml) or conditioned medium treated with P4 (10^{-7} M).

Growth curves and clonogenic assay

Primary HMECs were placed on collagen coated plates, 40,000 cells/well into 24 well plates or 100,000 cells/well into 6 well plates. Serum starvation was performed for 15h before starting the treatment with GH (10 ng/ml). Cells were harvested every two days and viable cells were counted using trypan blue staining. For clonogenic assay, cells were plated at 5,000 or 10,000/10 cm dish density in presence or absence of GH (10 ng/ml) and allowed to form colonies (14-16 days). Crystal violet staining was used to visualize colonies.

Table

Table S1

Primary Antibody	Company	Source	Working Dilution	Application	Secondary Antibody	Company	Working Dilution	Ag retrieval
ALDH1A1	BD Biosciences	Mouse	1:50	IF	Alexa Fluor 488 Donkey Anti-Mouse, Alexa Fluor 555 Donkey Anti-Mouse	Invitrogen	1:500	Citrate Buffer, pH6
ALDH1A1	BD Biosciences	Mouse	1:50	IHC	EnVision G2 Doublestain System Rabbit/Mouse (AEC)	DAKO		Citrate Buffer, pH6
ALDH1A3	Santa Cruz	Goat	1:100	IF	Alexa Fluor 555 Donkey Anti-Goat, Alexa Fluor 488 Donkey Anti-Goat	Invitrogen	1:500	Citrate Buffer, pH6
CD10-PE	BD Biosciences	Mouse	1:5	Flow				
CD10	Novocastra	Mouse	1:25	Flow	Anti-Mouse PE-Cy5 conjugated	BD Biosciences	1:200	
CD10	Novocastra	Mouse	1:25	IF	Alexa Fluor 488 Donkey Anti-Mouse, Alexa Fluor 555 Donkey Anti-Mouse	Invitrogen	1:500	
CD49f-PE-Cy5	BD Biosciences	Mouse	1:5	Flow				
CK14	Novocastra	Mouse	1:20	IF	Alexa Fluor 555 Donkey Anti-Mouse, Alexa Fluor 488 Donkey Anti-Mouse	Invitrogen	1:500	
CK18	Novocastra	Mouse	1:50	Flow	Alexa Fluor 555 Donkey Anti-Mouse	Invitrogen	1:1000	
CK18	Novocastra	Mouse	1:20	IHC	Peroxidase Histostain-Plus Kit	Zymed		Citrate Buffer, pH6
CK18	Novocastra	Mouse	1:20	IF	Anti-Mouse FITC conjugated, Anti-Mouse Texas Red conjugated	BD Biosciences	1:200	
ER	Novocastra	Rabbit	1:100	IHC	Peroxidase Histostain-Plus Kit	Zymed		Citrate Buffer, pH6
ER	Dako	Mouse	1:100	IHC	EnVision G2 Doublestain System Rabbit/Mouse (DAB)	DAKO		Citrate Buffer, pH6
ER	Neomarkers	Rabbit	1:100	Flow	Anti-Rabbit FITC conjugated	Jackson Labs	1:250	
EpCAM-FITC	emCell Technolog	Mouse	1:5	Flow				
ESA (EpCAM)	Novocastra	Mouse	1:25	Flow	Anti-Mouse PE conjugated	BD Biosciences	1:200	
GAPDH	Cell Signaling	Rabbit	1:5,000	WB	Peroxidase-conjugated Polyclonal Donkey Anti-Rabbit	Jackson Labs	1:250	
GH	Abcam	Rabbit	1:100	IF	Alexa Fluor 488 Donkey Anti-Rabbit	Invitrogen	1:500	Citrate Buffer, pH6
GH	Abcam	Rabbit	1:100	WB	Peroxidase-conjugated Polyclonal Donkey Anti-Rabbit	Jackson Labs	1:20,000	Citrate Buffer, pH6
GHR	Abcam	Rabbit	1:100	IHC	Peroxidase Histostain-Plus Kit	Zymed		Citrate Buffer, pH6
GHR	Sigma	Rabbit	1:100	Flow	Anti-Rabbit FITC conjugated, Anti-Rabbit PE conjugated	BD Biosciences	1:200	
ITGA6 (CD49f)	ATLAS	Rabbit	1:25	IF	Alexa Fluor 555 Donkey Anti-Rabbit	Invitrogen	1:500	Citrate Buffer, pH6
MCM2	Novocastra	Mouse	1:100	WB	Peroxidase-conjugated Polyclonal Donkey anti-Mouse	Jackson Labs	1:10,000	
MCM2	Novocastra	Mouse	1:50	IF	Alexa Fluor 555 Donkey anti-Mouse	Invitrogen	1:500	
Muc1	Novocastra	Rabbit	1:20	IF	Anti-Mouse FITC conjugated	BD Biosciences	1:200	
$p21^{WAF1/Cip1}$	DAKO	Mouse	1:100	WB	Peroxidase-conjugated Polyclonal Donkey Anti-Mouse	Jackson Labs	1:10,000	
PgR	DAKO	Mouse	1:100	IHC	Peroxidase-conjugated Polyclonal Donkey Anti-Mouse	Jackson Labs	1:10,000	
PgR	DAKO	Mouse	1:100	IF	Alexa Fluor 555 Donkey Anti-Mouse	Invitrogen	1:500	
PgR	DAKO	Mouse	1:1,000	WB	Peroxidase-conjugated Polyclonal Donkey Anti-Mouse	Jackson Labs	1:10,000	
SMA	Novocastra	Mouse	1:100	IHC	Peroxidase Histostain-Plus Kit	Zymed		Citrate Buffer, pH6
SMA	Novocastra	Mouse	1:100	Flow	Anti-Mouse PE conjugated	BD Biosciences	1:200	
Total Stat5	Cell Signaling	Rabbit	1:1,000	WB	Peroxidase-conjugated Polyclonal Donkey Anti-Rabbit	Jackson Labs	1:20,000	
PStat5(Tyr694)	Cell Signaling	Rabbit	1:1,000	WB	Peroxidase-conjugated Polyclonal Donkey Anti-Rabbit	Jackson Labs	1:20,000	
Tubulinβ	Cell Signaling	Rabbit	1:3,000	WB	Peroxidase-conjugated Polyclonal Donkey Anti-Rabbit	Jackson Labs	1:20,000	