Stem Cell Reports, Volume *4*

Supplemental Information

Disequilibrium of BMP2 Levels in the Breast Stem Cell Niche Launches Epithelial Transformation by Overamplifying BMPR1B Cell Response

Marion Chapellier, Elodie Bachelard-Cascales, Xenia Schmidt, Flora Clement, Isabelle Treilleux, Emmanuel Delay, Alexandre Jammot, Christine Menetrier-Caux, Gaëtan Pochon, Roger Besançon, Thibault Voeltzel, Claude Caron de Fromentel, Christophe Caux, Jean-Yves Blay, Richard Iggo, and Véronique Maguer-Satta

Supplemental information including supplemental experimental procedures and 5 figures.

Supplemental figures

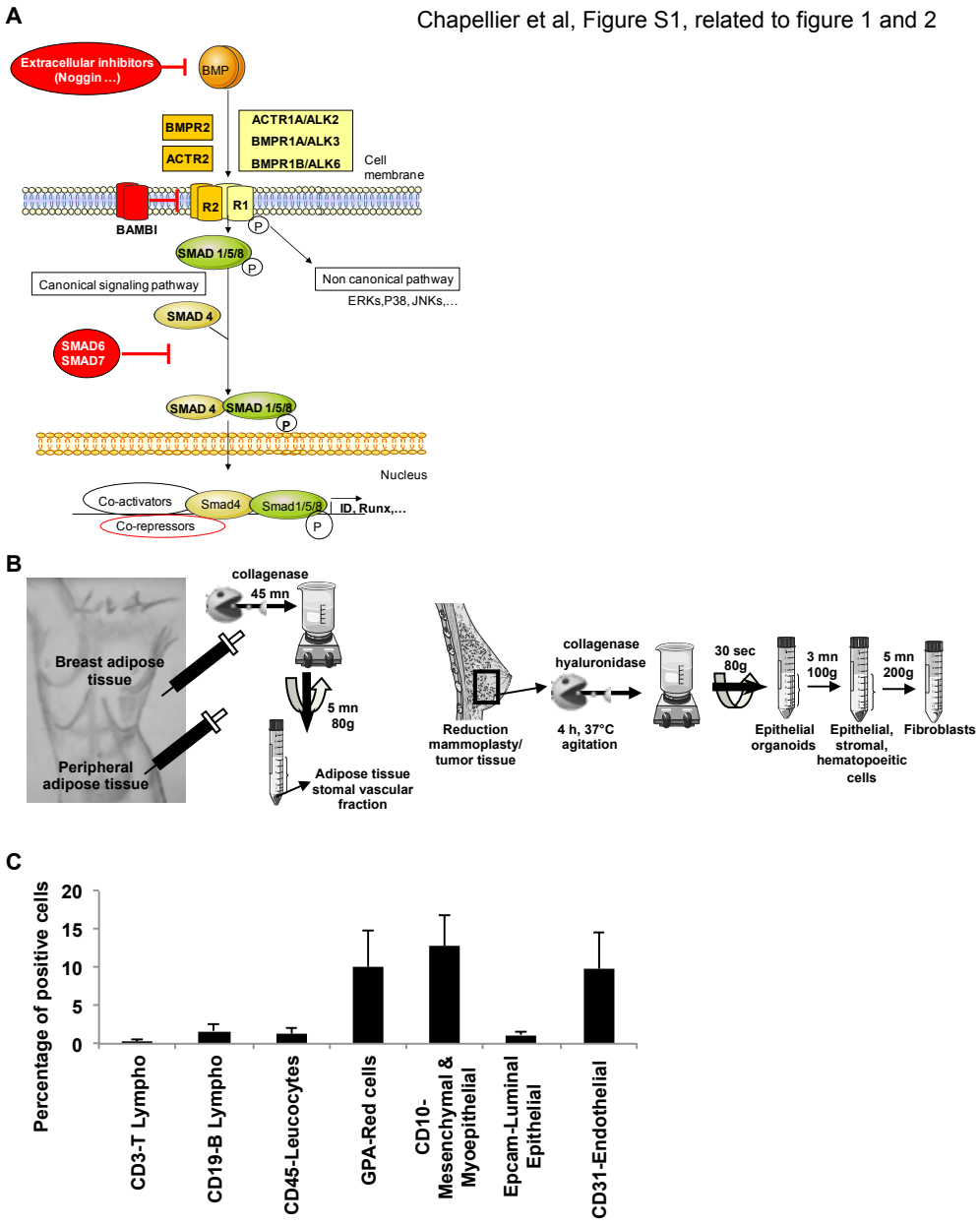


Figure S1. BMP signaling pathway and preparation of primary cells. (A) The canonical BMP signaling pathway is activated by soluble BMP ligands that bind to a complex of type 1 and 2 BMP receptors. Phosphorylated SMADs 1/5/9 form a complex with SMAD4 and activate target genes such as ID and RUNX. The pathway is regulated by extracellular inhibitors (Noggin), membrane-bound pseudoreceptors (BAMBI) and cytoplasmic inhibitors (SMAD6/7). Alternative pathways involve ERK, p38 and JNK kinases. (B) Left panel: adipose tissue samples were collected from lipo-aspirate from the peripheral mammary gland (breast adipose tissue) or from the abdomen (peripheral adipose tissue). Right panel: protocol to isolate distinct cell fractions enriched in either mammary stem cells (epithelial organoids), differentiated mammary epithelial cells, mixed stromal and hematopoietic cells and fibroblasts from reduction mammoplasty tissue, respectively. (C) Composition of the stromal and hematopoietic fraction described in (B) analyzed by flow cytometry for hematopoietic subpopulations: B (CD19) and T (CD3) lymphocytes, pan-leucocyte (CD45) and red blood (Glycophorin A-GPA); endothelial (CD31) and epithelial (EpCAM) cells. Results are presented as the mean of positive cells ± SEM, n=6 independent experiments.

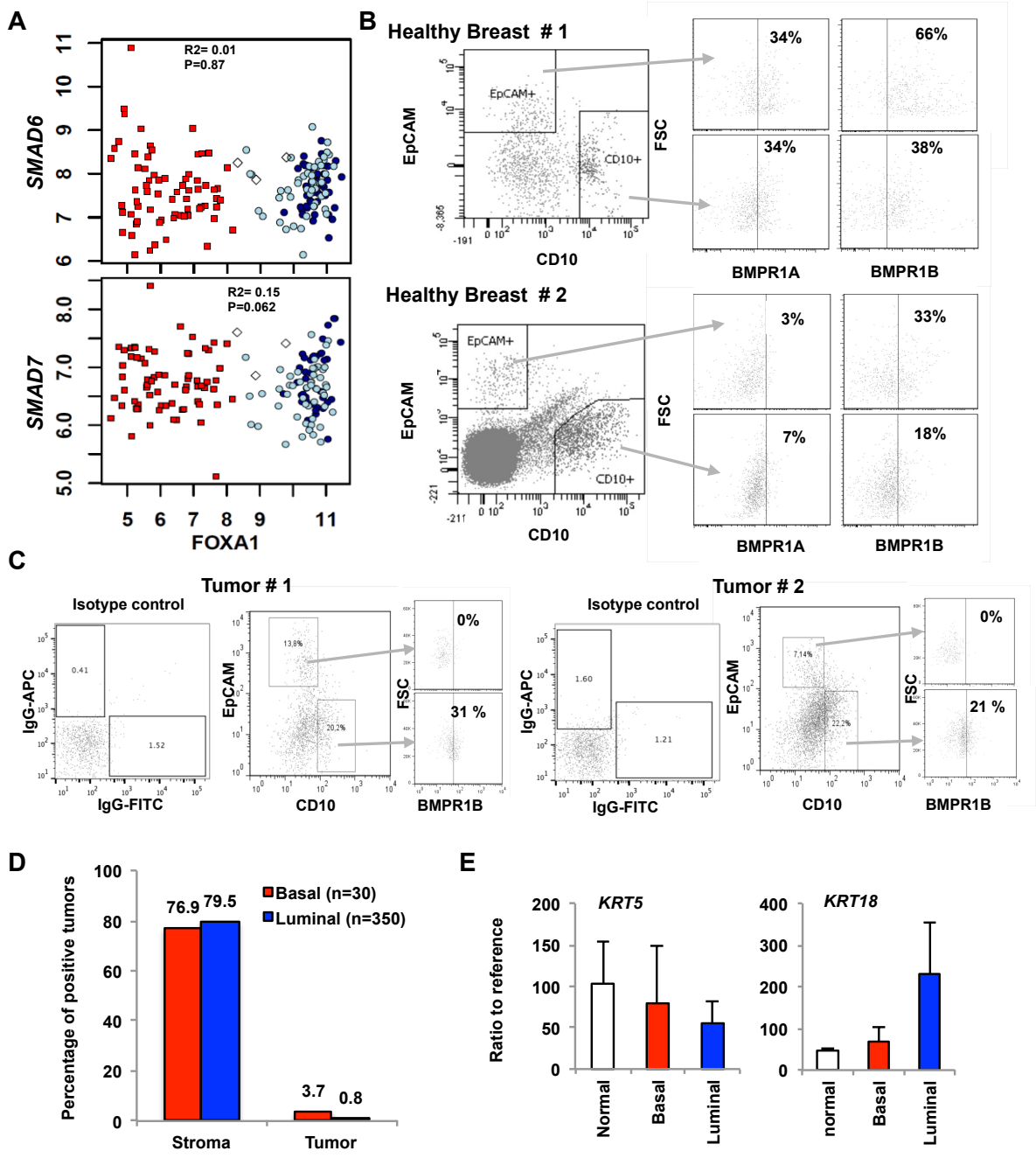


Figure S2. Deregulation of the BMP pathway in breast tumors. (A) Microarray analysis of normal and breast tumor samples for the expression of *FOXA1* versus *SMAD6* and *SMAD7*. White diamonds: normal samples, red squares: basal tumors, dark blue circles: luminal tumors, light blue circles: apocrine tumors. (B) BMPR1A and BMPR1B expression was analyzed by flow cytometry in CD10+ or EpCAM+ sorted fractions from 2 normal mammoplasty samples. (C) Flow cytometry analysis of BMPR1B expression in CD10+ or EpCAM+ sorted fractions from a luminal tumor sample. (D) IHC analysis of 440 tumors for CD10. The percentage of CD10-positive tumors for 30 basal and 350 luminal tumors that displayed an interpretable staining. (E) *KERATIN 5 (KRT5)* and *18 (KRT18)* expression in fresh normal tissue (*n*=10), luminal (*n*=16) and basal (*n*=4) breast tumor samples was analyzed by qPCR. Data represent the mean of the ratio to the expression in human mammaryfibroblasts ± SEM.

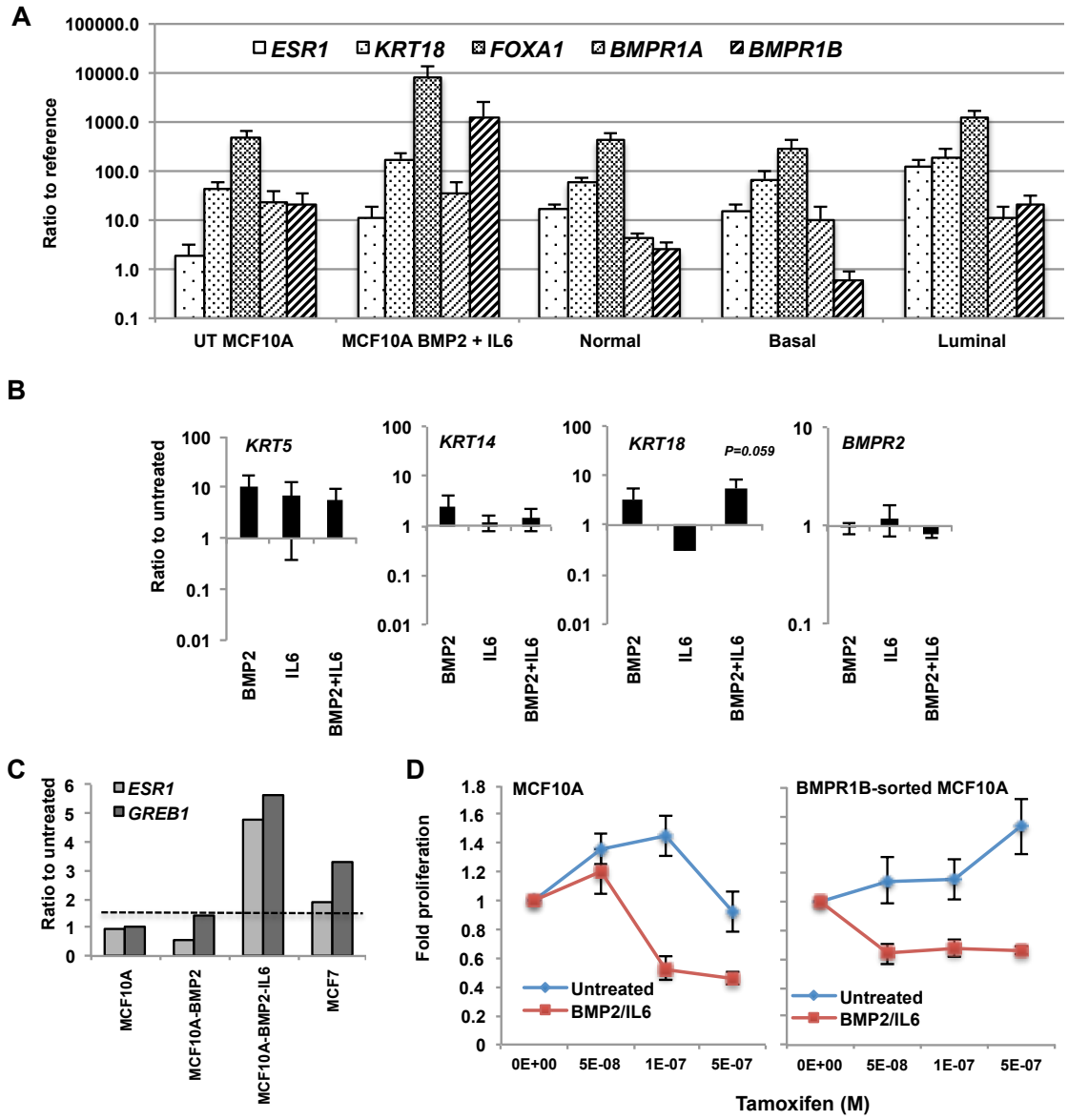
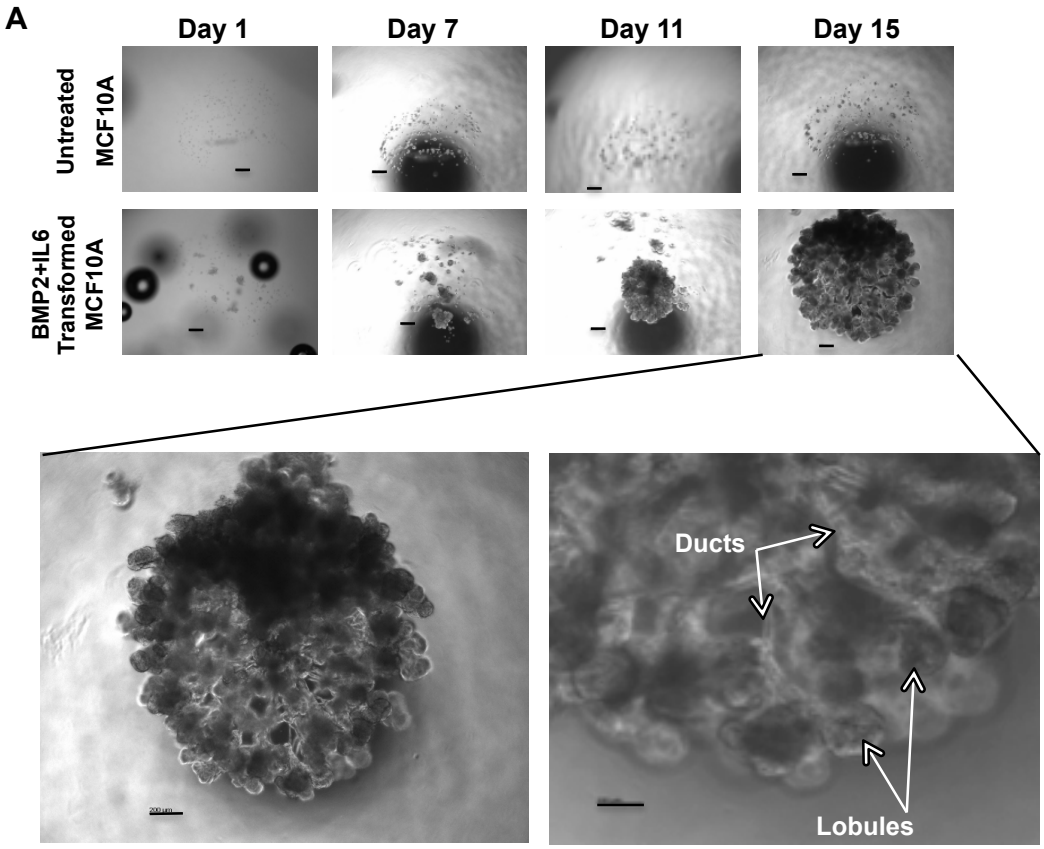


Figure S3. MCF10A treated with BMP2/IL6 display a luminal tumor profile with a functional estrogen response. (A) Comparison of MCF10A expression profile to primary normal and tumor tissue. qPCR analysis of *ESR1, Keratin 18 (K18), FOXA1, BMPR1A* and *BMPR1B* expression in MCF10A cells exposed to BMP2 ± IL6 for 10 weeks (n=6) and in normal (n=10), luminal (n=16) and basal (n=4) breast tumor samples. Data represent the mean ratio to reference fibroblast sample ±SEM. (B) qPCR analysis of *KRT5, 14* and *18* and *BMPR2* expression in MCF10A cells chronically exposed for 10 weeks to BMP2 +/- IL6. Data represent mean ratio to untreated cells ± SEM, *n*=6. (C, D) BMP2/IL6-treated MCF10A cells are responsive to 17 beta-estradiol (E2) treatment. (C) Untreated MCF10A control cells, MCF10A cells chronically exposed to BMP2 +/- IL6 for 20 months and MCF7 positive control cells were incubated in medium supplemented with delipidated serum for 24 hours and subsequently incubated in the absence or presence of 10^{-8}M E2 for 72 hours. Expression of the E2 responsive genes *ESR1* and *GREB1* was analyzed by qPCR. Results from one representative experiment are shown as the gene expression ratio of treated to untreated cells for each cell line. (D) Tamoxifen dose-response curves of treated MCF10A cells. Unsorted or BMPR1B+ MCF10A cells were exposed to BMP2 +/- IL6 for 6 months before culture in the presence of increasing doses of Tamoxifen for 6 days. Results represent mean ±SEM, n=3. (B-D) n indicates the number of independent experiments.



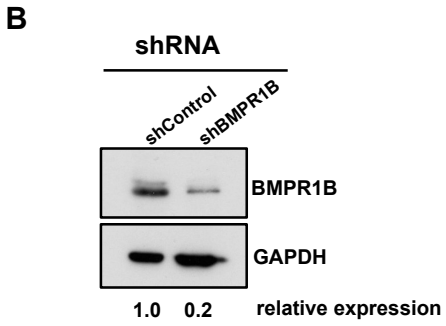


Figure S4. Functional characterization of BMP2/IL-treated MCF10A cells. (A) Growth of BMP2/IL6-treated MCF10A cells in 3D Terminal Duct Lobular Unit (TDLU) assay. 1E+03 MCF10A control or BMP2/IL6-treated cells were injected in a single point into growth factor-reduced matrigel following 20 months of long-term treatment. Upper panel: BMP2/IL6-treated cells form outgrowths with extensive branched structures. Unexpectedly, control MCF10A cells fail to form outgrowths, possibly du to prolonged culture. All scale bar represent 100 µm. Lower panel: Outgrowth of BMP2/6-treated cells at day 15 at higher magnification. Scale bar left 200 µm, right 100 µm. (C) Western Blot analysis confirming knockdown of BMPR1B expression using an shRNA lentiviral vector.

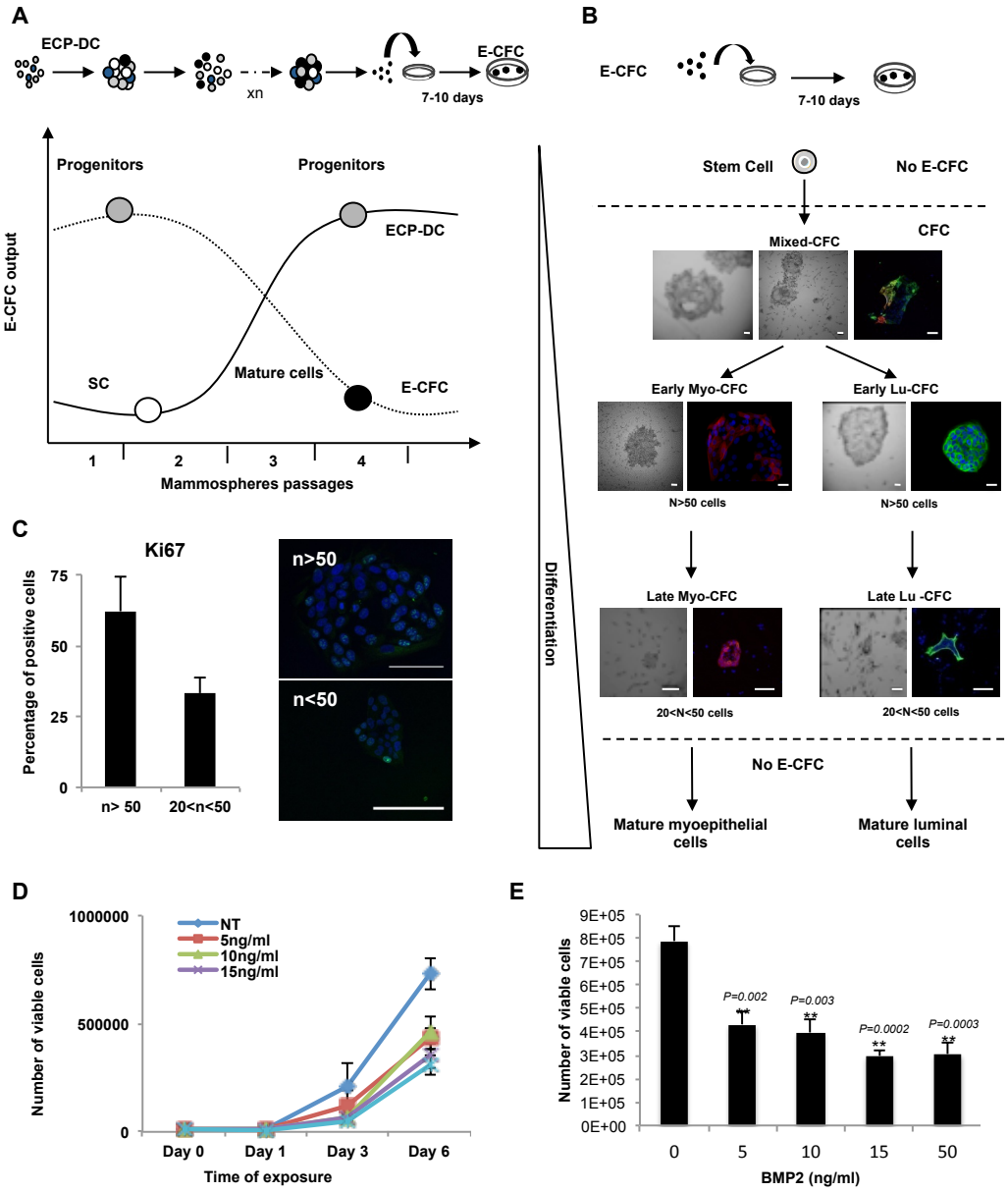


Figure S5. Functional characterization of mammary epithelial cells. (A) Schematic diagram showing the Early Common Progenitor-Derived Cell (ECP-DC) assay protocol. Epithelial cells were grown under non-adherent conditions, mammospheres were sequentially passaged and dissociated cells were analyzed using the E-CFC (Epithelial Colony Forming Cell) assay that relects the stem cell cell content at initiation of the ECP-DC assay (Bachelard-Cascales et al., 2012). (B) Protocol illustrating the E-CFC assay for the quantification of epithelial progenitors. Epithelial cells are plated at a limiting density on a layer of irradiated NIH3T3 feeder cells. After one week, colonies are fixed and counted according to their morphology and size as illustrated in the bright-field images of the different types of colonies that reflect epithelial cell subtypes of different lineages. Luminal or myoepithelial/basal status is confirmed by keratin 18 (green) or keratin 14 (red) immunofluorescence, respectively. E-CFC: Epithelial-Colony Forming Cell, Myo-CFC: Myoepithelial-CFC, Lu-CFC: Luminal-CFC. Scale bar 200 µm. (C) Percentages of Ki67 positive cells in E-CFC (>50 cells and 20<n<50 cells) and immunofluorescence imaging of Ki67 staining (green) inside E-CFC nucleus. Scale bar=100µM. (D) BMP2 dose-response curve for MCF10A cells. Cells were cultured for 6 days. Data represent the mean cell number *(n=5* independent experiments*)* ± SEM. (E) The same dose-reponse data represented as number of cells at day 6.

Supplemental experiment procedures

Preparation of single cell suspensions from mammary tissue samples

The culture of human tissue samples was approved by the ethics board of the Leon Berard Cancer Center, and patients gave informed consent. Primary mammary epithelial cells and adipocytes were isolated from human adult tissue as described (Bachelard-Cascales et al., 2010). Briefly, normal breast tissue from reduction mammoplasties was digested with 0.8 mg/ml collagenase and 15 U/ml hyaluronidase (Sigma-Aldrich) for 3-5 hours at 37°C. The epithelial cell-rich pellet (pellet A) was collected by centrifugation for 30 sec at 80 x g. The supernatant was centrifuged for 3 min at 100 x g to collect a pellet enriched in stromal and hematopoietic cells (Pellet B), the second supernatant was centrifuged for 5 min at 200 x g to collect mammary fibroblasts (Pellet C) (Figure S1B). The first epithelial cell-rich pellet was dissociated by 10 min digestion in 0.05% trypsin-EDTA solution (Invitrogen), centrifuged (80 x g, 10 min), resuspended and digested for 10 min in 5 mg/ml of dispase and 1 µg/ml DNase I (Sigma-Aldrich), centrifuged (80 x g, 10 min) and filtered using 70 µM and 40 µM cell strainers (Falcon). Primary mammary epithelial cells were cultured as described in Experimental Procedures. Human mammary fibroblasts were cultures in DMEM medium (Life Technologies) supplemented with 10% FBS or phenol red-free DMEM/F12 nutrient mixture supplemented with 5% FBS.

Preparation of single cell suspensions from adipose tissue

Adipose tissue samples were obtained from liposuction samples digested with collagenase at 0.4 mg/ml for 45 min at 37°C, 5% CO_2 to eliminate fibroblasts. The reaction was stopped by addition of DMEM/F-12 nutrient mix (Invitrogen), supplemented with 5% fetal bovine serum (BioWhittaker). The cell suspension was separated by Pancoll (PAN-Biotech) density gradient centrifugation, and the upper phase containing lipids were eliminated and cells were resuspended in DMEM/F12 nutrient mix (Life Technologies) supplemented with 2% bovine serum albumine (Stemcell Technology). The cell suspension was filtrated using a 70 µm cell strainer. Adipose tissue samples were cultured in phenol red-free DMEM/F12 nutrient mixture supplemented with 5% FBS.

Terminal Duct and Lobular Unit (TDLU) assay

Terminal duct lobular unit (TDLU) assays were performed as described (Bachelard-Cascales et al., 2010). Briefly, Lab-Tek chamber slides (Nunc) were coated with 300 µl/cm^2 growth factor-reduced Matrigel Matrix (Corning) and Matrigel was allowed to harden for 2-3 min. 1,000 MCF10A cells in a volume of 1-2 µl were injected into the semi-hardened matrigel in one point and placed at 37°C, 5% CO_2 for 30 min. Complete medium was added, and medium was changed every 3-4 days. Analysis of 3D structures and all other assays were performed using Axiovert 25 microscope (Zeiss), and images were taken with a Power shot A640 camera and analyzed with AxioVision 4.6 software.

BMP and carcinogen treatment

For the E-CFC and ECP-DC assays, primary mammary cells were treated by adding 50 ng/ml of BMP2 and BMP4 (R&D Systems) to complete culture medium supplemented with 2% horse serum at the indicated time. For long-term treatment of MCF10A cells, BMP2 or

IL6 (Peprotech) were added at a concentration of 10 ng/ml. To assess the effect of carcinogenic agents, primary cells isolated from different normal mammary tissue fractions and adipose tissue (Figure S1B) were cultured in phenol red-free DMEM/F12 nutrient mix supplemented with 5% FBS for two days. 24 hours prior to treatment, medium was changed to DMEM/F12 nutrient mix supplemented with 5% delipidated FBS. Cells were treated with 10^{-6} M Bisphenol A (BPA), 10^{-6} M Bisphenol S (BPS), 10^{-10} M Benzo[a]pyrene (BaP) (all Sigma-Aldrich) or irradiated at 7 Gray. Supernatants and cells were harvested after 4 days of treatment. BMP2 expression was quantified by qPCR, and protein concentrations were measured by ELISA.

Lentiviral vectors and infections

The CMV-BMP2-mPGK-hygromycin lentiviral vector construct and its corresponding control were a gift from Dr R. Iggo, University of Bordeaux, France. The pLenti X2 Puro empty control vector (#20957) and the pLenti X2 puro DEST (#17296) used to clone the pX2-shBMPR1B vector were purchased from Addgene (Campeau et al, 2009). Lentiviruses were produced by calcium phosphate cotransfection of lentiviral constructs with a VSV-G envelope construct (pMD2.G) and gagpol packaging construct (PCMVdR8.74) into HEK 293T cells according to standard techniques (Dull et al., 1998; Follenzi and Naldini, 2002). 6 hours post transfection the medium was replaced. Lentiviral particles were collected 48 hours post transfection. Lentiviral titers were determined for each viral batch by serial dilution infections of MCF10A cells and subsequent puromycin or hygromycin (both Sigma-Aldrich) treatment. MCF10A cells were seeded one day prior to infection and cells were infected overnight at a multiplicity of infection of 5-10. 48 hours post infection, transduced cells were selected by puromycin or hygromycin B treatment for 96 hours to two weeks.

Flow cytometry and cell sorting

Cells were resuspended in HBSS, 2% FBS and incubated for 30 minutes to 1 hour with 0.1 g of the following IgG1 antibodies per 10^6 cells: PE- or FITC-or APC-or Pe-Cy5-conjugated IgG1 isotype control, PE-conjugated or FITC-conjugated anti-CD10 (BD Biosciences), APC-conjugated anti-EpCAM (BD Biosciences), Pe-Cy5-conjugated anti-CD49f (BD Biosciences), FITC-conjugated anti-BMPR1A (R&D systems) or PE-conjugated anti-BMPR1B (R&D systems) antibodies. After centrifugation, cells were resuspended in HBSS, 2% FBS for flow cytometry cell sorting at a concentration of 5–10.10^6 cells/ml. Cell sorting was performed using a FACS Aria cell sorter (BD Biosciences) at low pressure (psi: 20) with 488 nm and 633 nm lasers. For phenotypic analysis, cells were labeled by incubation with antibodies, washed and fixed with 2% formaldehyde solution (Sigma-Aldrich) prior to flow cytometry analysis using a FACSCalibur Cell Analyzer (BD Biosciences).

Soft agar colony formation assay

To evaluate the transformation of cells, soft agar colony formation assays were performed as follows. The bottom agar layer was prepared from 1.5% agar (Promega) diluted in an equal volume of 2X culture medium to a final concentration of 0.75%, added to cell culture plates and incubated at room temperature for 30 min. The top agar layer was prepared accordingly at a final concentration of 0.45%. Cells were mixed into the liquid top agar and added on top of the bottom agar at a final concentration of 15,000 cells/ml. Cell culture plates were

incubated at room temperature for 30 min and covered with medium. Colonies were quantified and measured following 3-5-weeks of culture at 5% CO_2 and 37°C.

Xenografts

Animal experiments were authorized by the ethics committee for animal experimentation of the Rhone-Alpes region (CECCAPP), France. Following long-term treatment with treated with BMP2 and IL6, two million or five million MCF10A shControl or shBMPR1B cells, respectively, were mixed with 50% growth factor-reduced Matrigel (BD Biosciences) and injected subcutaneously in proximity of the forth inguinal mammary gland of 6-7-week-old athymic nude mice (Harlan). 5 mice were injected per group. A 10 mg/ml beta-estradiol solution was applied to the neck region of the animals twice a week. Tumor formation was followed by size measurement. Mice were sacrificed after 6 weeks, and tumors were fixed, paraffin-embedded, sectioned and subjected to H&E staining.

Immunofluorescent staining

Primary mammary or MCF10A cells were grown on glass cover slips in multi-well cell culture plates (BD Biosciences), fixed with 4% paraformaldehyde (PAF) for 15 minutes, permeabilized with 0.1% Triton X-100 (Sigma-Aldrich) for 10 min, and blocked with 0.2% gelatin (Sigma-Aldrich) at room temperature for 30 min. The cells were then incubated for 1 hour at room temperature with mouse monoclonal anti-Cytokeratin 18 (AbCys) using ready-to-use antibody diluent (DAKO), washed twice before incubation with a secondary Alexa Fluor 488-conjugated goat anti-mouse antibody (Life Technologies Molecular Probes). Cells were washed twice and incubated for 1 hour at room temperature with an AffiniPure Fab fragment donkey anti-mouse IgG. Following washing, cells were incubated in 0.2% gelatin for 20 min, followed by incubation with a mouse monoclonal anti-Cytokeratin 14 antibody (AbCys) for 1 hour at room temperature. Finally, cells were washed twice and incubated with Alexa Fluor 647-conjugated goat anti-mouse secondary antibody (Life Technologies Molecular Probes) for 30 min. Controls for nonspecificity and autofluorescence were performed by incubating cells with either no antibody, the primary antibody alone, or the secondary antibody alone. For Ki67 staining, cells were incubated with Ki67-FITC antibody (BD Biosciences) for 1h according to the manufacturer's instructions following fixation and permeabilization. Analysis of stained cells was performed using a Zeiss LSM 780 confocal microscope.

Western blot analysis

Whole cell extracts were prepared by lysis of cells in 2X protein loading buffer (62.5 mM Tris-HCl, pH 6.8, 10% glycerol, 2% SDS, 5% β-mercaptoethanol, 0.01% bromophenolblue) or modified radioimmunoprecipitation assay buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% IGEPAL CA-630, 0.25% sodium deoxycholate, 1 mM EDTA) supplemented with protease and phosphatase inhibitor cocktails (Roche Applied Science). Equal amounts of total protein were separated by SDS polyacrylamid gel electrophoresis. Western blotting was carried out according to standard techniques. Primary antibodies directed against BMPR1B (Abcam) or GAPDH (Abcam) and secondary antibodies (Jackson Immuno Research) were diluted in 5% non-fat milk in Tris-buffered saline, 0.1% Tween-20 (Sigma).

Quantitative real-time PCR analysis

RNA was extracted using the RNeasy Plus Mini Kit (Qiagen) containing a gDNA eliminator column or TriReagent (Sigma-Aldrich) and chloroform extraction using Phase Lock Gel columns (5Prime, Hilden, Germany). RNA concentrations were determined using a Nanodrop ND-1000 spectrophotometer (Thermo Scientific, Wilmington, USA). Reverse transcription was carried out using Superscript II reverse transcriptase (Life Technologies) according to the manufacturer's instructions. qPCR was performed using the primers detailed below on a LightCycler 480 II system (Roche Applied Science) with the QuantiFAST SyBR kit (Qiagen). CPB and ACTB1 were selected as reference genes by geNorm analysis (Vandesompele et al., 2002). A pool of cDNAs from three normal human primary fibroblast samples was used as a reference sample to normalize expression levels.

Gene	Accession number	Sense primer	Antisense primer
ACTB	NM_001101	5'-ATTGGCAATGAGCGGTTC-3'	5'-GGATGCCACAGGACTCCAT-3'
BMP2	NM_001200	5'-AGACCTGTATCGCAGGCACT-3'	5'-CCTCCGTGGGGATAGAACTT-3'
BMP4	NM_001202	5'-CTTTACCGGCTTCAGTCTGG -3'	5'-GGGATGCTGCTGAGGTTAAA-3'
BMPR1A	NM_004329	5'-GAAAAAGTGGCGGTGAAAGT-3'	5'-TAGAGCTGAGTCCAGGAACC-3'
BMPR1B	NM_001203	5'-GCCAGCTGGTTCAGAGAGAC-3'	5'-CAGGACCCTGTCCCTTTGAT-3'
BMPR2	NM_001204	5'-TAGCACCTGCTATGGCCTTT-3'	5'-CTGAATTGAGGGAGGAGTGG-3'
CPB	NM_000942.4	5'-ACTTCACCAGGGGAGATGG-3'	5'-AGCCGTTGGTGTCTTTGC-3'
ESR1	NM_001122742	5'-TTACTGACCAACCTGGCAGA-3'	5'-ATCATGGAGGGTCAAATCCA-3'
FOXA1	NM_004496	5'-GAAGATGGAAGGGCATGAAA-3'	5'-CGCTCGTAGTCATGGTGTTC-3'
FOXC1	NM_001453	5'-AGCGCAGAACTTCCACTCG-3'	5'-GGAAGGCCATTTGACAGCTA-3'
FST	NM_006350	5'-TCTGCCAGTTCATGGAGGAC-3'	5'-CCCGTTGAAAATCATCCACT-3'
GATA3	NM_002051	5'-GAGCCCCTACTCGCCCTAC-3'	5'-CAGGCGTTGCACAGGTAGT-3'
GREB1	NM_014668	5'- TGCCAACAACTGGCAAAGAA-3'	5'-CACCCTTTGTGGCGTTTTTT-3'
K14	NM_000526	5'-TGACCTGGAGATGCAGATTG-3-	5'-CATACTGGTCACGCATCTCG-3'
K18	NM_000224	5'-CCAGTCTGTGGAGAACGACA-3'	5'-CTGAGATTTGGGGGCATCTA-3'
NOG	NM_005450	5'-GGCCAGCACTATCTCCACAT-3'	5'ATGAAGCCTGGGTCGTAGTG-3'
SMAD1	NM_005900	5'-CGCGTTCCTTCTGAAAATTG-3'	5-TGCAAAAGGACAGCAGAAGA-3'
SMAD5	NM_001001420	5'-TCTGCTTGGGTTTGTTGTCA-3'	5'-GCAGCTGCTGGGAATCTTAC-3'
SMAD6	NM_005585	5'-CTGCAACCCCTACCACTTCA-3'	5'-AGAATTCACCCGGAGCAGT-3'

ACTB, Beta Actin; BMP, Bone Morphogenetic Protein; BMPR, Bone Morphogenetic Protein Receptor; CBP, cyclophilin B (peptidylprolyl isomerase B, PPIB); ESR1, estrogen receptor 1; FOXA1, Forkhead box A1; FOXC1, Forkhead box C1; FST, Follistatin; GATA3, GATA binding protein 3; GREB1, Growth Regulation by Estrogen in Breast cancer 1; K14, keratin

14 (KRT14); K18, keratin 18 (KRT18); NOG, noggin; SMAD, Small Mothers Against Decapentaplegic homolog.

Statistical analysis

Treated cells were compared to untreated cells by paired Student's t-tests using α= 0.05.

References

Bachelard-Cascales, E., Chapellier, M., Delay, E., and Maguer-Satta, V. (2012). A protocol to quantify mammary early common progenitors from long-term mammosphere culture. Current protocols in stem cell biology *Chapter 1*, Unit 1E 7.

Bachelard-Cascales, E., Chapellier, M., Delay, E., Pochon, G., Voeltzel, T., Puisieux, A., Caron, d.F., and Maguer-Satta, V. (2010). The CD10 enzyme is a key player to identify and regulate human mammary stem cells. Stem Cells *28*, 1081-1088.

Campeau, E., Ruhl, V.E., Rodier, F., Smith, C.L., Rahmberg, B.L., Fuss, J.O., Campisi, J., Yaswen, P., Cooper, P.K., and Kaufman, P.D. (2009). A versatile viral system for expression and depletion of proteins in mammalian cells. PloS one *4*, e6529.

Dull, T., Zufferey, R., Kelly, M., et al. (1998). A third-generation lentivirus vector with a conditional packaging system. J Virol 72, 8463-8471.

Farmer, P., Bonnefoi, H., Becette, V., Tubiana-Hulin, M., Fumoleau, P., Larsimont, D., Macgrogan, G., Bergh, J., Cameron, D., Goldstein, D*., et al.* (2005). Identification of molecular apocrine breast tumours by microarray analysis. Oncogene *24*, 4660-4671.

Follenzi, A., and Naldini, L. (2002). Generation of HIV-1 derived lentiviral vectors. Methods Enzymol 346, 454-465.

Vandesompele, J., De Preter, K., Pattyn, F., Poppe, B., Van Roy, N., De Paepe, A., and Speleman, F. (2002). Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome biology *3*, RESEARCH0034.